

**PREPARATION OF HUMAN MYOMETRIUM FOR TERM:
THE ROLE OF SIGNALLING ASSOCIATED PROTEINS**

**By
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The study presented in this thesis is entirely the product of my own work.

Panadda Hatthachote

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DEDICATION

This thesis is dedicated to all the Thai people whose sacrifice has given me the opportunity to study abroad. I would especially like to thank the children from rural Thailand who have contributed to my education although they may not have received adequate schooling themselves.

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PUBLICATIONS

Full Papers

HATTHACHOTE, P., MORGAN, J., DUNLOP, W., EUROPE-FINNER, G.N. & GILLESPIE J.I. (1998) Gestational changes in the levels of transforming growth factor- β 1 (TGF β 1) and TGF β receptor types I and II in the human myometrium. *Journal of Clinical Endocrinology and Metabolism* **83**, 2987-2992.

HATTHACHOTE, P. & GILLESPIE J.I. (1999) Complex interactions between sex steroids and cytokines in the human pregnant myometrium: evidence for an autocrine signalling system at term. *Endocrinology* **140**, 2533-2540.

Abstracts

MORGAN, J., AWAD, S.S., HATTHACHOTE, P., DUNLOP, W. & GILLESPIE, J.I. (1997) Up regulation of intracellular Ca²⁺ signalling systems in the human pregnant myometrium: a role for TGF β 1. *Acta Obstetricia Et Gynaecologica Scandinavica* **76**, Suppl 167:2, 31.

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TABLE OF CONTENTS

Chapter 1 INTRODUCTION	1
1.1 The initiation of human labour	1
1.1 Pre-term labour	4
1.3 Quiescence and contractility in the pregnant myometrium	5
1.4 Regulation of myometrial function during pregnancy	6
1.4.1 Role of contraction-associated proteins (CAPs) and myometrial activation	6
1.4.2 Role of stimulatory agents and uterine stimulation	11
1.5 Regulation of contraction-associated proteins	14
1.6 The contribution of cytokines to human pregnancy and parturition	16
1.7 Cytokines and possible roles in normal pregnancy	18
1.7.1 IL-1 and IL-1 receptors	18
1.7.2 IL-8 and IL-8 receptors	19
1.7.3 TGF β and TGF β receptors	21
1.8 Regulation of the levels of TGFβ mRNA, TGFβ secretion and activation	24
1.8.1 Role of sex steroid hormones	24
1.8.2 Role of cAMP	26
1.8.3 Role of cytokines	27
1.9 Choice of cytokines	28
AIMS OF THE STUDY	29
Chapter 2 METHODS	30
2.1 Myometrial collection and ethical approval	30
2.1.1 Myometrial collection	30
2.1.2 Ethical approval	31
2.1.3 Removal of myometrium from the uterus	31
2.2 Preparation of human myometrial lysate	31
2.3 Immunohistochemistry	32
2.3.1 Tissue section determination of TGF β receptors in human myometrial smooth muscle	32

2.3.2 Indirect immunostaining method	32
2.4 Human myometrial cell culture techniques	33
2.4.1 Isolation of human myometrial smooth muscle cells	33
2.4.2 Cell passage	34
2.4.3 Estimation of cell numbers	34
2.4.4 Verification of myometrial smooth muscle cell purity	35
2.5 Treatment of cultured myometrial smooth muscle cells with Cytokines, steroid hormones, antibodies to cytokines and forskolin	38
2.5.1 IL-1 and IL-8 treatment and determination of TGF β 1 and T β RI expression	38
2.5.2 TGF β 1 and IL-8 treatment and determination of T β RI and IL-8RB	39
2.5.3 Sex steroid hormones treatment and determination of TGF β 1 and T β RI	39
2.5.4 Cytokines and cytokines neutralising antibodies treatment and determination of T β RI	39
2.5.5 Cytokines and cytokines neutralising antibodies treatment and determination of cyclooxygenase-2 (COX-2)	40
2.5.6 Forskolin treatment and determination of TGF β 1, T β RI, c-Fos and c-Jun	40
2.5.7 TGF β 1 treatment and determination of the G protein sub-units (G α_s and G $\alpha_{i,2}$)	41
2.6 Total protein assay	41
2.7 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting	42
2.7.1 Protein sample preparation and gel electrophoresis	42
2.7.2 Western Blotting: wet blotting system	42
2.8 Immunoblotting and enhanced chemiluminescence system (ECL)	43
2.9 Quantification of immunoreactive bands	45
2.10 TGFβ1 immunoassay	49
2.10.1 Principle of the assay	49
2.10.2 Sample preparation	51
2.10.3 Activation procedures	51
2.10.4 Preparation of standards	51
2.10.5 Assay procedures	52
2.10.6 Calculation of results	53
2.11 Intracellular cAMP enzyme-immunoassay	53
2.11.1 Sample preparation	53

Appendix 2	145
Appendix 3	147
Appendix 4	148
Appendix 5	149

Chapter 1

INTRODUCTION

1.1 The initiation of human labour

Much of our knowledge of parturition stems from “the progesterone block” theory of labour, which was first proposed by Csapo in 1961 (see Greer, 1995). This theory suggests that there is a balance between myometrial stimulants, including oestrogen and prostaglandins, and myometrial relaxants such as progesterone. During pregnancy this balance would be tipped in favour of progesterone but at parturition progesterone would fall and the balance tip in favour of myometrial stimulants. Although this theory is true for parturition in sheep it is not the case for humans where there is no change in progesterone concentration at the time of labour. The endocrinological events associated with the initiation of parturition are much better understood in ovine than in human pregnancies (for review see Cunningham *et al.* 1997; Nathanielsz, 1999). Pioneering work of Liggins *et al.* (1973) showed that in the sheep, the fetus was responsible for determining the timing of labour. It was demonstrated in these studies that fetal hypophysectomy or bilateral fetal adrenalectomy led to prolonged pregnancy. Contrarily, the infusion of adrenocorticotropin or cortisol into the sheep fetus earlier in pregnancy caused pre-term delivery. Thus, it is likely that fetal pituitary-adrenal axis is involved in the initiation of parturition in sheep

The postulated role of the pituitary-adrenal axis was strengthened by the demonstration of a rise in the concentration of cortisol in the fetal lamb circulation during the last 15 days of pregnancy (Liggins, 1973). Cortisol is produced by a cascade mechanism whereby the fetal hypothalamus releases corticotropin releasing factor (CRF) which in turn stimulates adrenocorticotrophic hormone (ACTH) in the pituitary. ACTH in turn stimulates adrenal cortisol production, which acts in the placenta to stimulate activity of 17-hydroxylase, which is required to convert progesterone to oestrogen (France *et al.* 1988). Thus, the balance between myometrial relaxation and myometrial contraction is disturbed with a

reduction in progesterone and an increase in oestrogen. This change in placental steroid production thereby stimulates prostaglandin production via an increased activity of prostaglandin synthetase, gap junction formation, myometrial excitability and enhancement of uterine sensitivity to oxytocin.

A role for the fetus in the initiation of primate parturition is also suggested by the findings of studies conducted in the rhesus monkey. It has been shown that fetal hypophysectomy can prolong gestation. If the fetus is removed, with the placenta left in situ, spontaneous delivery of the placenta is delayed (Nathanielsz *et al*, 1992). It can be interpreted from this study that the normal signal to deliver does not occur in the absence of the fetus.

In the human fetus, the pituitary is necessary for adrenal maturation and steroidogenesis during the second half of pregnancy. However, there is little evidence of a role for fetal cortisol in the initiation of human labour. The major difference in endocrine events between these species is that the human placenta lacks 17-hydroxylase. Therefore, no changes in progesterone or oestrogen levels are found at the onset of labour (Liggins, 1983). Infusion of glucocorticoids to a human fetus failed to induce parturition (cited by Challis and Lye, 1994) and furthermore mothers with an anencephalic fetuses went into spontaneous labour, although the length of gestation is more variable than in normal pregnancies (Lye, 1996). Despite the suggestion that the hypothalamo-pituitary-adrenal axis in the human fetus is unable to alter the steroid hormones at the placental level, there is evidence to support activation of this axis, which may play a central role in co-ordinating the transition from a state of quiescence to one of contraction. Maternal plasma levels of CRF increase dramatically during the third trimester (Grammatopoulos & Hillhouse, 1999). Furthermore, high levels of CRF have also been associated with the onset of pre-term labour (Frim *et al*. 1988). The source of CRF is not from fetus or maternal hypothalamus but from the placenta (Frim *et al*. 1988). It has been suggested that placental CRF can stimulate the fetal adrenal to produce cortisol and dihydro-epiandrosterone (DHEA)-sulphate, the latter being

converted to oestradiol by placental aromatase, and thus facilitate uterine activity (Greer, 1995)

It seems that there is evidence implicating a role for the pituitary-adrenal axis in human labour. However, human labour cannot be induced by cortisol administration and it is known that cortisol inhibits prostaglandin production by inhibiting the action of phospholipases (Blackwell *et al.* 1986). In addition, the observation from recent *in vitro* studies has shown that CRH did not affect myometrial tension development (Simpkin *et al.* 1999). Therefore, CRH is unlikely to play a major role in the onset of labour and in the control of myometrial contractility. Other factors must therefore be involved in human parturition. It has been proposed that paracrine and autocrine mechanisms play a central role in the control of human parturition. The observations that locally acting prostaglandins are involved in parturition and increased prostaglandins in the uterus support a role for the uterine environment in this process. It is also likely that there is no single factor able to initiate the onset of human labour, but rather a parturition cascade that remove the mechanisms maintaining uterine relaxation and recruits factors promoting uterine activity (Lye, 1996; Norwitz *et al.* 1999). These agents are called uterotonins, such as oxytocin and prostaglandin (see section 1.4.2). It is now recognised that the expression of these potent stimulants is increased in intra-uterine tissues during late pregnancy (Chibbar *et al.* 1993). Once the myometrium is prepared, endocrine, paracrine and autocrine factors then induce a switch from irregular contraction to co-ordinated contraction at term.

Dysfunctional labour and disorders of parturition such as pre-term labour are a major cause of obstetric intervention. Thus, a better understanding of the physiological processes responsible for the onset and control of labour at term will advance our knowledge and improve our ability to develop better treatments to ensure a successful birth.

1.2 Pre-term Labour

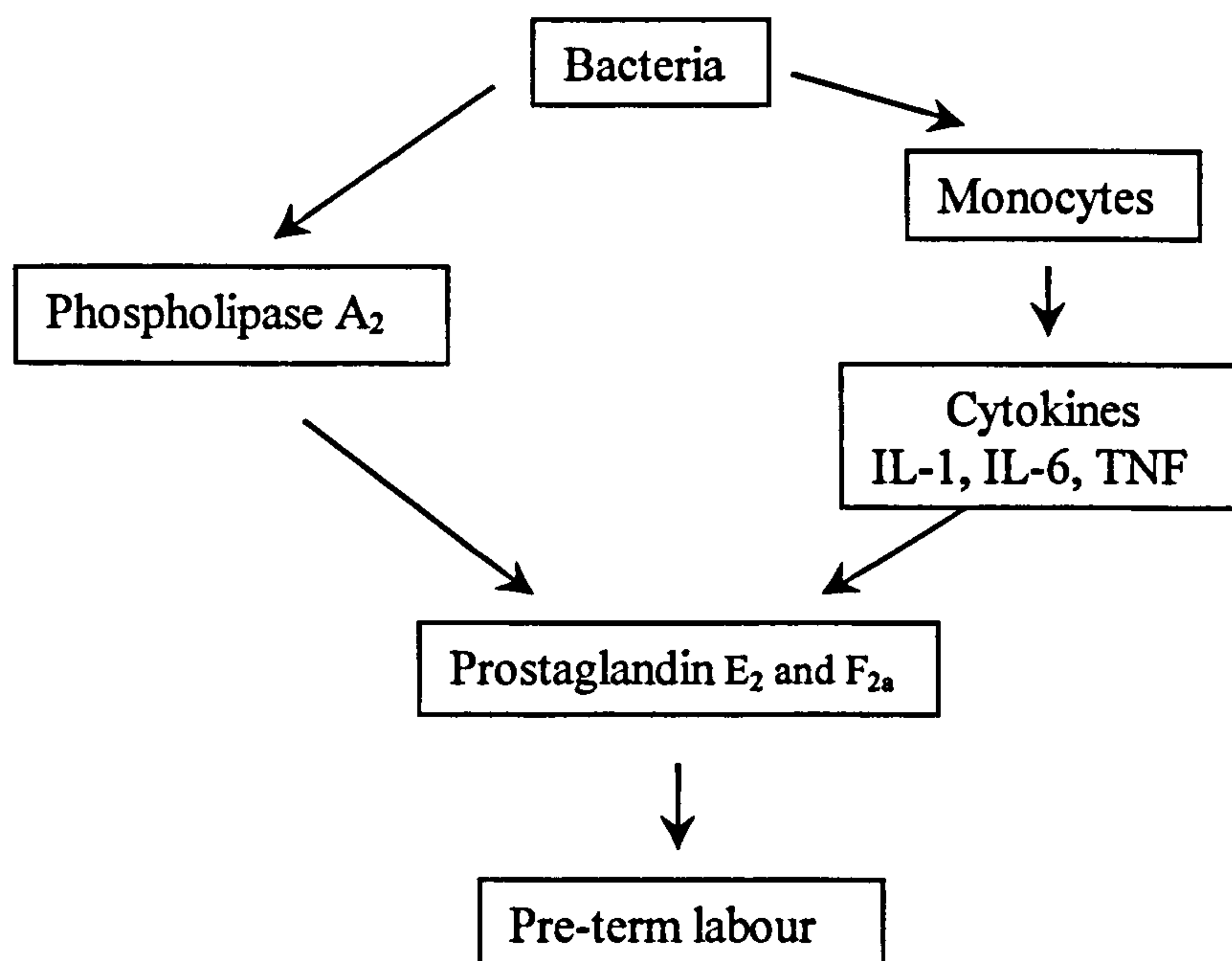
Pre-term labour, defined as labour before 37 weeks of gestation, occurs in up to 10 % of all births and is associated with 85 % of all perinatal complications and death (Norwitz *et al.* 1999). The costs consume more than one-third of health care expenditure during the first year of life. Moreover, additional expenditure for handicaps is necessary during the remainder of childhood for many infants. A wide spectrum of causes, including multiple gestation, uterine anomalies, infection, smoking, and demographic factors such as maternal age, race and poverty have been implicated in pre-term birth (Cunningham *et al.* 1997; Norwitz *et al.* 1999). Up to 30 % of pre-term labours result from intraamniotic infection (Romero & Mazor, 1988). Prostaglandins are considered to play a significant part in the onset and progression of labour both at term and pre-term (Thorburn & Challis, 1979).

Bejar *et al.* (1981) reported that many microorganisms produce phospholipase A₂, which cleaves arachidonic acid within fetal membranes, thereby making free arachidonic acid available for prostaglandin synthesis. This may potentially initiate pre-term labour. It has been reported that introduction of bacterial endotoxin (lipopolysaccharides) in the amniotic fluid stimulates decidual cells to produce prostaglandin (Mitchell *et al.* 1991b) and hence the early onset of labour.

Pre-term parturition due to infection is thought to be initiated by endogenous secretory products, cytokines, resulting from monocyte activation. In many patients with infection, there are increased levels of cytokines including interleukin-1 (IL-1), IL-6 and tumour necrosis factor- α (TNF- α) in the amniotic fluid. These cytokines have been implicated in pre-term labour as increased prostaglandin synthesis has been observed in many cell types following treatment with these cytokines (for review see Mitchell *et al.* 1993b). A simplified mechanism of action by which bacteria induce pre-term labour is shown below.

Prematurity and its consequences remain a major health problem. Although the mortality following pre-term birth has fallen the long-term consequences have not improved.

The prevention of pre-term labour has been very difficult because of poor understanding and the inability to predict myometrial contractility. Several biochemical markers such as activin, inhibin and fibronectin (Goldenberg *et al.* 1996; Petralgia, 1997) have been associated with pre-term labour. However, only fetal fibronectin in cervicovaginal secretions, which may reflect separation of the fetal membranes from the maternal decidua has been used successfully as a screening test for pre-term delivery (Goldenberg *et al.* 1996). New drugs specific to the mechanism of contractility with fewer unacceptable side effects need to be developed to prevent labour or prolong pregnancy until a stage where the fetus has an increased chance of survival.



1.3 Quiescence and contractility in the pregnant myometrium

During pregnancy, the myometrium is maintained in a quiescent state by mechanisms, which limit myometrial contractility. These mechanisms include the maintenance of elevated plasma or myometrial levels of progesterone, the involvement of other hormones such as relaxin, catecholamines and nitric oxide, and the expression of G-protein in the myometrium

(for reviews see Garfield & Yallampalli, 1994; Lopez Bernal *et al.* 1995; Liu & Rebar, 1999). Late in pregnancy, these mechanisms become less effective, meanwhile, other processes essential for initiation of contraction appear and become dominate. At which point during late pregnancy when these processes occur is unknown. However, it can be thought that sometime near term and before the onset of labour, these processes appear and the myometrium is prepared for effective contraction (Figure 1.1).

By which processes myometrial contraction is initiated in human are not well understood. It has been suggested that an increase in myometrial activity and contractility during late pregnancy result from the myometrium being activated by synthesis and expression of a cassette of key proteins involving in contractile events. These proteins collectively were called contraction-associated proteins (CAPs) (Lye, 1994, 1996). Once activated, the myometrium is capable of responding to the uterotonic agents such as oxytocin and prostaglandin (Lye, 1996). Then, the onset of labour begins.

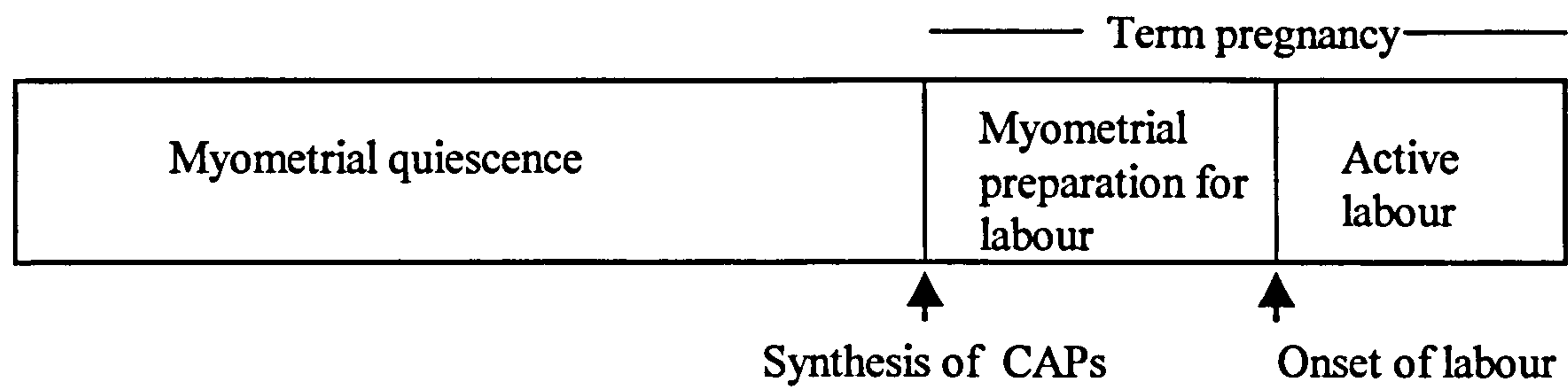


Figure 1.1 Stages of pregnancy

1.4 Regulation of myometrial function during pregnancy

1.4.1 Role of contraction-associated proteins (CAPs) and myometrial activation

Gap junctions, ion channels, uterotonin (e.g., oxytocin, prostaglandins) receptors and endothelin receptors are recognised to be CAPs which are present in the myometrium at term (Boyle & Heslip, 1994; Chow & Lye, 1994; Mershon *et al.* 1994; Kimura *et al.* 1996; Wolff *et al.* 1996; Brodt-Eppley & Myatt, 1999). Awad *et al* (1997) has suggested that the

intracellular Ca^{2+} release mechanism (RyR2), whose levels of gene expression increase in the pregnant myometrium during late gestation, is another example of a CAP. Once these proteins are expressed within the myometrial smooth muscle, the myometrium is transformed from an overall state of quiescence to one primed for effective contraction.

Gap junctions

Gap junctions are channels composed of proteins called connexins (Willecke *et al.* 1991). Gap junctions provide pathways for the movement of ions, second messengers (Ca^{2+} and inositol 1,4,5-trisphosphate (IP_3)) and for the flow of current between cells. Therefore, gap junctions enhance electrical coupling and facilitate the spread of excitation (Tabb & Garfield, 1992; Lopez Bernal, 1996). In the myometrium, a 43-kDa protein identified as connexin 43 (Cx-43) is known to be the major component of a gap junction (Fishman *et al.* 1990; Lang *et al.* 1991).

Gap junctions are not detected in human myometrium in the early stages of pregnancy (Andersen *et al.* 1993) but are present in myometrial tissues obtained from women in labour undergoing caesarean section compared with those not in labour at term (Garfield & Hayashi, 1981). The elevation of gap junction expression is found to be associated with an increase in Cx-43 mRNA and protein expression in the myometrium (Chow & Lye, 1994). Recently, it has been reported that levels of Cx-43 protein in the human myometrium at term are increased in upper segment myometrial samples compared to those in lower segment (Sparey *et al.* 1999). In addition, Cx-43 levels in the myometrium during spontaneous labour are higher than that of non-labouring myometrium (Sparey *et al.* 1999). These results concur with the report by Chow and Lye (1994) indicating Cx-43 is increased in the human myometrium toward term and with the onset of labour. It has been suggested that the differential expression of Cx-43 may underlie the propagation of contractions from the fundus to the cervix during the course of labour (Sparey *et al.* 1999).

Ca²⁺ channels

It is known that smooth muscle cells contract in response to a rise in $[Ca^{2+}]_i$ and relax as the $[Ca^{2+}]_i$ fall. The level of $[Ca^{2+}]_i$ can be raised by an influx of Ca^{2+} through the plasma membrane either via voltage-dependent Ca^{2+} channels (VOC) or through receptor-operated channel (ROC). Ca^{2+} can also be released from intracellular stores, for example the sarcoplasmic reticulum (SR) (Taylor & Traynor, 1995).

Two types of VOC, T-type and dihydropyridine (DHP)-sensitive L-type Ca^{2+} channels, have been described in the human myometrium (Young *et al.* 1993). It has been suggested by Batra (1989) that there is no difference in the binding of dihydropyridine between pregnant and non-pregnant myometrium. However, in rat myometrium, mRNA encoding the L-type Ca^{2+} channel increases during late pregnancy before term (Tezuka *et al.* 1995) and the spliced forms of the L-type channel increase during labour (Mershon *et al.* 1994).

The SR is considered to be the most important store responsible for regulation of cytoplasmic Ca^{2+} levels. The evidence so far suggests that there are two Ca^{2+} release channels on the SR: the IP_3R - Ca^{2+} release channel and the Ca^{2+} induced- Ca^{2+} release channel (RyR) (Berridge, 1993; Taylor & Traynor, 1995; Bezprozvanny & Ehrlich, 1996). At least five IP_3R isoforms, encoded by different genes, exist on the SR in many cell types (Danoff *et al.* 1991; De Smedt *et al.* 1994). A 220-kDa IP_3R type I isoform has been found in human myometrium (Yamada *et al.* 1994).

A second type of SR Ca^{2+} release channel, the RyR, is activated when $[Ca^{2+}]_i$ increases, and this produces the phenomenon of Ca^{2+} -induced Ca^{2+} release (CICR). Three genes encoding for RyRs have been identified; the RyR1 gene encoding the skeletal muscle channel, the RyR2 gene present in cardiac muscle and RyR3 expressed in brain (Sorrentino & Volpe, 1993; Meissner, 1994). It has been thought that myometrium probably does not express the RyRs, and hence CICR is unlikely. This conclusion was based on the observation that caffeine, the usual probe for the RyR-CICR mechanism, was unable to elevate $[Ca^{2+}]_i$ in the myometrium (Kanmura *et al.* 1988). It has been suggested that this

conclusion is incorrect because a novel type of CICR system, which is ryanodine-sensitive but caffeine-insensitive (RyR3), have been found in isolated human myometrial cells (Lynn *et al.* 1993). Continuing work has confirmed that RyR3 isoform is expressed in cultured myometrial smooth muscle cells and in both non-pregnant and pregnant human myometrium. Interestingly, RyR2 is found only in pregnant non-labouring myometrium (Awad *et al.* 1997). Based on these observations, the RyR2 system appears to be one of the contraction-associated proteins that is up-regulated in human myometrium at term. The functional significance of the RyR2 in human myometrium during pregnancy is not known. However, the upregulation of this gene may control $[Ca^{2+}]_i$ levels and amplify the Ca^{2+} transient needed to initiate contraction. Therefore, the myometrium can increase its ability to produce powerful contractions at term (Awad *et al.* 1997).

Oxytocin receptor

Both messenger ribonucleic acid (mRNA) and protein for oxytocin receptor are present in the non-pregnant myometrium at low levels. The expression levels of oxytocin receptors in the myometrium are found to increase significantly just before the onset of labour and reach the peak at parturition (Kimura *et al.* 1996). It has been found that the oxytocin receptors are distributed unevenly throughout the myometrium. The oxytocin receptors in the fundus, corpus and upper part of the lower segment are almost uniformly distributed. In the lower part of the lower segment, the levels of oxytocin receptor diminish and become very low in cervical tissue (Fuchs *et al.* 1984). It is well documented that uterine sensitivity to oxytocin gradually increases with advancing gestational age, accelerating during the last few days before the onset of labour (Husslein & Leitich, 1995). The rise in uterine responsiveness to oxytocin correlates with an increase in oxytocin receptor concentrations in the human myometrium (Fuchs *et al.* 1984; Husslein & Leitich, 1995). Thus, the increase in oxytocin receptors allows oxytocin to trigger contractions without a rise in plasma levels of oxytocin.

Prostaglandin receptor

There is substantial evidence to suggest the presence of multiple sub-types of PG receptors in human myometrium (Hofmann *et al.* 1983; Adelantado *et al.* 1988; Matsumoto *et al.* 1997; Brodt-Eppley & Myatt, 1999). Upon binding to their ligands, different intracellular pathways are activated, depending on receptor subtypes. Pharmacologically, prostaglandin E (EP₁, EP₃), prostaglandin F (FP) and thromboxane (TX) are linked to contractile events. EP₂, EP₄, prostaglandin I and D (IP and DP) are associated with relaxation (Coleman *et al.* 1994). It has been reported that contractile receptors (EP₃ and FP) are down regulated during pregnancy (Matsumoto *et al.* 1997). Recently, EP₂ receptor mRNA expression has been found to increase during pregnancy and to decrease towards term, whereas FP receptor expression increases dramatically at term with labour (Brodt-Eppley & Myatt, 1999). Taken together, it can be suggested that down regulation of contractile prostaglandin receptors may influence the maintenance of uterine quiescence. In addition, an increase in contractile receptor concentrations in human myometrium at term may mediate myometrial contraction. As the concentration of EP receptors does not change during menstrual cycle (Hofmann *et al.* 1983; Adelantado *et al.* 1988), it is unlikely that EP receptors are controlled by ovarian steroids.

Endothelin receptor

It is generally accepted that endothelin is one of several receptors participating in the control of uterine activation. Receptors for endothelin type A (ET_A) and type B (ET_B) have been found in human myometrium (Wolff *et al.* 1996). ET_A receptor is involved in myometrial contractility by coupling to phospholipase C to generate IP₃ and inducing an increase in [Ca²⁺]_i (Heluy *et al.* 1995). It has been shown that ET_A receptor expression is higher in the upper than in the lower uterine segment and the ET_B receptor is expressed in the opposite pattern (Wolff *et al.* 1996). ET_A receptor mRNA in the upper uterine segment is significantly increased at term pregnancy, indicating a role of ET_A receptor in parturition. The physiological function of the ET_B receptor is unknown. However, it has been suggested

that this receptor may mediate myometrial relaxation as it can stimulate nitric oxide release *in vitro* (Yallampalli *et al.* 1993)

1.4.2 Role of stimulatory agents and uterine stimulation

Oxytocin

Oxytocin is a hormone secreted from the posterior pituitary gland. It has been discovered that oxytocin may be synthesised in uterine tissue itself (Lefebvre *et al.* 1992). Oxytocin stimulates myometrial contraction during labour by two parallel mechanisms: (1) by binding to cell membrane receptors coupled to G-proteins (Kimura *et al.* 1992), increasing $[Ca^{2+}]_i$ via the inositide phospholipid pathway (Phaneuf *et al.* 1993) and (2) by stimulation through increasing PG release (Fuchs *et al.* 1982). Although oxytocin is used to induce labour and control postpartum haemorrhage, the precise role for oxytocin in the initiation of parturition remains unclear. This is because the concentration of oxytocin in maternal plasma does not change significantly before and during labour (Dawood *et al.* 1978; Leake *et al.* 1981) and the progress of labour is not related to an increase in circulating oxytocin concentrations (Thornton *et al.* 1992). It has been argued that oxytocin receptor expression in the myometrium may be so great that oxytocin action can occur with only small levels of circulating oxytocin. It is evident that the oxytocin receptors increase dramatically around the onset of labour (Fuchs *et al.* 1984; Kimura *et al.* 1992). Thus, the physiological importance of oxytocin in the initiation of labour could be triggered by an increase in myometrial sensitivity to oxytocin rather than the increase in circulating concentrations of this hormone. Another possibility might be that local production of oxytocin in the uterus might act in an autocrine or paracrine manner to stimulate contraction. This mechanism would diminish the responsibility of the circulating oxytocin. Oxytocin has been found to stimulate PG synthesis (Fuchs *et al.* 1982), thus, oxytocin may interact with other uterine stimulants to regulate contractility and improve myometrial contraction. In spite of the uncertain role for oxytocin in the initiation of labour, oxytocin remains an important hormone related to uterine function.

Prostaglandins (PGs)

PGs have been proposed as a stimulus for myometrial contraction during parturition in normal labour and in infection-induced pre-term labour (Kelly, 1994). The lines of evidence supporting PGs as being important in the initiation of spontaneous labour include the following:- PG levels in maternal plasma and amniotic fluid rise with labour progression and administration of prostaglandin E and F (PGE₂ and PGF_{2α}) at any stage of pregnancy induces labour (Olson *et al.* 1995). In addition, successful pregnancy has been found to be associated with a rise in prostaglandin metabolites in the maternal plasma and in amniotic fluid (Sellers *et al.* 1982). All uterine tissues, including myometrium, amnion, chorion and decidua are capable of producing PGs from substrate arachidonic acid (Fuchs & Fuchs, 1996). Therefore, there is no doubt that PG synthesis is involved in human parturition. PG actions are mediated by specific receptors (see section 1.4.1) that link to multiple mechanisms depending on receptor subtypes (Lye, 1994; Lopez Bernal *et al.* 1995). Such diversity in signalling may explain the conflicting observation of the response to PG in the uterus. PGF_{2α} elevates [Ca²⁺]_i both by opening Ca²⁺ channels and by the release of Ca²⁺ from intracellular stores by the activation of phospholipase C (PLC) (Phaneuf *et al.* 1993), and hence stimulates myometrial contraction (Fuchs & Fuchs, 1996). PGE₂ activates PLC followed by elevating of [Ca²⁺]_i and activating of MLCK (MacKenzie *et al.* 1990). However, it also increases cAMP production (Lopez Bernal *et al.* 1991), dependent on EP receptor type.

PGs are formed from arachidonic acid released from cell membranes. Enzymes involved in this initial step are PLC and phospholipase A₂. Free arachidonic acid is converted to PG through the activity of prostaglandin H synthase, or cyclooxygenase (COX) (Rosen *et al.* 1989). Two isoforms of COX have been found. COX-1 is expressed constitutively in most types of tissue whereas COX-2 is expressed only following cell activation by cytokines and mitogens (see DeWitt, 1991).

While synthesis of PGs is regulated initially by activation of phospholipases, PG production is also dependent on the level of expression of COX (DeWitt, 1991). COX-1 and COX-2 have been identified in amnion (Hirst *et al.* 1995). There is no change in COX-1 expression whereas the expression of COX-2 has been shown to increase in amnion after the onset of labour (Hirst *et al.* 1995), suggesting that COX-2 is responsible for PG synthesis in amnion. The factors that control the expression of COX-2 remain to be identified, but interleukin-1 (IL-1) has been shown to increase COX-2 mRNA expression in cultured amnion cells (Mitchell *et al.* 1993a).

In human myometrium, COX-1 and COX-2 are located in the myometrial smooth muscle (Zuo *et al.* 1994). The expression of COX-2 mRNA and protein in the myometrium increases at term and decreases during labour for both pre-term and term pregnancy. In contrast, COX-1 expression is lower at term compared to pre-term and not dependent on labour (Zuo *et al.* 1994). From the same study, the amounts of PGE₂ and PGF_{2α}, have been found by immunostaining to be lower in term compared to pre-term and the amounts are further decreased with labour. A recent study by Moore *et al* (1999) has shown that the levels of COX-1 mRNA expression appears higher at term before labour compared to pre-term. However, the difference is not significant. For COX-2 mRNA, the levels of expression in the myometrium did not change either with gestational age or with labour. Sparey *et al* (1999) has reported that the levels of expression of both COX-1 and COX-2 proteins are not affected by the onset of parturition. In addition, higher levels of expression of these enzymes are demonstrated in the lower compared to the upper uterine segment. In contrast to these works, Slater *et al* has reported the up-regulation of COX-2 mRNA and protein in the human myometrium at term but no significant differences are observed in association with labour. Taken together, although there is no consistency in the expression of COX-2, it indicates an alteration in the cyclooxygenase pathway in the myometrium during pregnancy. Since COX-2 is inducible and short-lived, it is more likely to be restricted to particular areas

of activation (Kelly, 1994). Thus, myometrium obtained from different areas may provide different observations.

1.5 Regulation of contraction-associated proteins

It is now believed that myometrial contractility can be controlled by the synthesis of the CAPs and uterotonic agents. This concept leads to the physiological questions “what are the signals and processes regulating the synthesis and expression of these genes and proteins which subsequent influence myometrial function and what is the origin of the signals? ”. At present, the signals controlling the expression of the CAPs are poorly understood.

As gap junctions are amongst of the CAPs whose expression increases dramatically just prior to the onset of labour, regulation of gap junction expression may reflect the expression of other CAPs. Although the regulation of gap junction formation has been studied extensively, the mechanisms that regulate the synthesis of gap junctions are still under investigation. Based on studies in animals, the expression of Cx-43 mRNA and protein are regulated negatively by progesterone and positively by oestrogen (Petrocelli & Lye, 1993; Hendrix *et al.* 1995). Progesterone suppresses Cx-43 gene transcription and inhibits the transport of the Cx-43 protein out of the Golgi vesicles (Hendrix *et al.* 1995). Oestrogen can induce increases in Cx-43 mRNA through the synthesis of c-fos and c-jun genes and c-Fos and c-Jun proteins in the myometrium, which would in turn activate an activating protein-1 (AP-1) binding site in the Cx-43 promoter (Lefebvre *et al.* 1995; Piersanti & Lye, 1995).

In isolated human myometrial cells, oestrogen and progesterone have also been shown to affect the levels of Cx-43 expression (Andersen *et al.* 1993; Zhao *et al.* 1996). Oestrogen causes an increase in the levels of Cx-43 mRNA and protein expression (Andersen *et al.* 1993) and progesterone is found to decrease the transcription of Cx-43 mRNA (Andersen *et al.* 1993; Zhao *et al.* 1996). In contrast to animals, c-Jun and c-Fos, the factors that bind to AP-1 sites in the human Cx-43 promoter, are not involved in the mechanism by which

oestrogen increases Cx-43 expression in human myometrial cell culture (Geimonen *et al.* 1998). However, c-Fos and c-Jun proteins are found to be increased in the pregnant myometrium that also have elevated Cx-43 expression, suggesting an involvement of these proteins in the induction of Cx-43 expression at term in human myometrium (Geimonen *et al.* 1998). In addition to steroid hormones, protein kinase C (PKC) has been reported to induce Cx-43 mRNA expression followed by an increase in Cx-43 protein levels in human myometrial smooth muscle cells through an up-regulation of c-Fos and c-Jun proteins (Geimonen *et al.* 1996).

Sex steroid hormones have also been shown to have a role in the regulation of oxytocin receptor levels. In animals, oestrogen increases the levels of oxytocin receptors in the myometrium whereas progesterone reduces their levels (Nissenson *et al.* 1978). In human, oestrogen action on the oxytocin receptor probably has the same effect. Progesterone action may be different as there is evidence of an increase in the levels of oxytocin receptors in the myometrium at term when plasma progesterone levels are high (Fuchs *et al.* 1984; Kimura *et al.* 1996). An alteration in the oxytocin receptor levels has led Kimura *et al.* (1996) to suggest that regulation of oxytocin receptor expression is involved in the process of uterine preparation for labour.

Little is known about the processes involved in the synthesis and expression of other CAPs in the human myometrium. There is evidence to show that transforming growth factor- β 1 (TGF β 1) can modify levels of RyR2 expression in a number of cell types (Giannini *et al.* 1992; Neylon *et al.* 1994). Awad *et al.* (1997) has demonstrated that TGF β 1 induces the expression of RyR2 mRNA in cultured myometrial smooth muscle cells. The authors have suggested that TGF β 1 may serve as a signal in the myometrium and in term of RyR2 expression, TGF β 1-treated cells are similar to pregnant myometrium whose RyR2 isoform is up-regulated.

The expression of cyclooxygenases in human myometrial cells may also be regulated by Interleukin (IL)-1 β (Hertelendy *et al.* 1999). This results in an increase in PGE₂ and PGF_{2 α} production. Based on these *in vitro* experiments, it can be hypothesised that cytokines such as TGF β 1 and IL-1 are involved in the maturation of the myometrium *in vivo* during late pregnancy.

1.6. The contribution of cytokines to human pregnancy and parturition

There is evidence that intrauterine infection increases production of cytokines by gestational tissue, leads to PG synthesis and being followed by pre-term labour (Mitchell *et al.* 1993b; Mazor *et al.* 1995). Although there is much evidence supporting the role of cytokines in pre-term labour induced by infection, the evidence for their participation in the initiation of normal labour is sparse. Fundamentally, term and pre-term labour share a common terminal pathway consisting of uterine contractility, cervical dilatation and rupture of membranes (Mazor *et al.* 1995). This has led to the idea that there may be an association between cytokines and normal labour.

Cytokines are a diverse group of polypeptides, secreted in very small amounts by all nucleated cell types and involved in a variety of actions. The activities of cytokines are mediated via cell surface receptors. Thus, cytokine activity can be regulated either by controlling the availability of the cytokines or the expression of receptors. One cytokine may have different effects, depending on the target cells. However, most cytokine effects involve an altered pattern of gene expression in target cells (Mazor *et al.* 1995).

It is increasingly apparent that intrauterine infection is associated with pre-term labour and delivery (Romero & Mazor, 1988; Romero *et al.* 1994). The basis for this concept is the fact that bacterial infection in decidua and fetal membranes can stimulate cytokine release by the immune system. Bacterial products as well as cytokines can then induce prostaglandin synthesis in human decidua, amnion and chorion (Lamont *et al.* 1985). Cytokines such as interleukin 1 (IL-1) and tumour necrosis factor alpha (TNF α) can

stimulate PG production in many cell types (Mazor *et al.* 1995). It has been confirmed that in human decidua and chorion, these cytokines can enhance the synthesis of PG, which may initiate the onset of labour and subsequent delivery the fetus (Norwitz *et al.* 1992; Pollard *et al.* 1993). In human myometrial cells, IL-1 and TNF stimulate arachidonic acid release, and PG production (Molnar *et al.* 1993).

Other cytokines whose levels are increased in amniotic fluid of patients with pre-term labour in relation to intrauterine infection include IL-6 and IL-10 (Romero *et al.* 1993; Greig *et al.* 1995). Although much interest has focused on the potential role of cytokines in the induction of pre-term labour, they may also be of significant importance in the physiological process of parturition. This idea arises from studies by Gunn *et al.* (1996) and Stallmach *et al.* (1995) showing that the concentrations of IL-1, IL-6 and IL-8 are high in amniotic fluid and maternal plasma with normal pregnancy. It is also found that normal spontaneous labour at term is associated with a moderate rise in the levels of TNF α , IL-1 and IL-6 in amniotic fluid (Saito *et al.* 1993) and IL-6 in maternal plasma (Arntzen *et al.* 1997). IL-1 in amniotic fluid may be secreted from stimulated monocytes or neutrophils in decidua, which are recruited to the amniotic space (Cunningham *et al.* 1993). It has also been reported that keratinocytes, which are abundant in amniotic fluid, may be a source of amniotic IL-1 (Romero *et al.* 1989a). TGF β has also been found in amniotic fluid and plasma during pregnancy. There is no change in total acid released transforming growth factor- β (TGF β)-1 levels in plasma during pregnancy (Wakefield *et al.* 1995) and in amniotic fluid in term delivery (Mazor *et al.* 1995). However, the concentrations of TGF β are also increased in human amniotic fluid with pre-term labour and delivery but regardless of intrauterine infection (Mazor *et al.* 1995). Thus, the significance of this cytokine remains to be established.

1.7 Cytokines and possible roles in normal pregnancy

1.7.1 IL-1 and IL-1 receptors

In most systems, two forms of IL-1, IL-1 α and IL-1 β , are synthesised (see Colotta *et al.* 1998). Their biological actions of these two forms are indistinguishable, and the mature forms of IL-1 act by way of receptors, type I (IL-1RI) and type II (IL-IRII). IL-1 is particularly synthesised in macrophages and monocytes. It is also produced in other tissues in response to specific stimuli. Stimuli for IL-1 production include toxins from bacteria or bacterial products, phorbol esters and other cytokines such as TNF α , interferons and IL-1 itself. The biological actions of IL-1 are numerous and are directed at a wide range of target cells. Briefly, the role of IL-1 can be defined as the initiation of the defense reaction. IL-1 is produced at the site of inflammation. It stimulates surrounding cells such as monocytes and lymphocytes to exert its defense actions. Thus, IL-1 initiates a defensive mechanism and triggers the immune function (Dinarello, 1991). Consistent with a central role in host defence, IL-1 can induce PG synthesis by decidua, amnion and chorion (Romero *et al.* 1989b; Pollard *et al.* 1993), as well as several cytokines including IL-8 and IL-1 itself (Dinarello, 1989). Considering the stimulatory effect of IL-1, it is likely that IL-1 forms a major step in the gestational inflammatory network (DeJongh *et al.* 1996).

Human decidua and placenta are known to be sources of IL-1 (Romero *et al.* 1989c; Taniguchi *et al.* 1991; Ammala *et al.* 1997). IL-1 β mRNA expression and protein levels in decidua are higher than in placenta (Ammala *et al.* 1997). Comparing decidual samples from non-labouring and spontaneous labouring pregnancies, IL-1 β mRNA expression and protein levels are higher in labouring samples than in non-labouring samples. IL-1 β has been found recently in the lower segment of human myometrium at term in which the concentration is increased during labour and parallel to cervical dilatation (Winkler *et al.* 1998). In addition, there is no increase in the level of IL-1 β after 6 cm of dilation. In the same study, it has been found that the level of IL-8 in the myometrium changes parallel to the IL-1 β level (Winkler

et al. 1998). Thus, it has been hypothesised that IL-1 β may be involved in an induction of IL-8 in the lower segment of human myometrium

IL-1 receptors have been demonstrated in decidua, placenta and fetal membranes (Ammala *et al.* 1997; Whittle *et al.* 1999). Therefore, IL-1 may act in an autocrine fashion in decidua and placenta. IL-1RI mRNA expression is stronger in decidua than in placenta (Ammala *et al.* 1997). In decidua, both mRNA and proteins for IL-1RI and IL-1RII are expressed. In the fetal membranes, only IL-1RII has been found (Whittle *et al.* 1999). Interestingly, the pattern of receptor expression in decidua does not change with labour (Whittle *et al.* 1999). Specific binding sites for IL-1 have been demonstrated in human myometrial cells in culture (Hertelendy *et al.* 1993). However, there is no report on the expression levels of IL-1 receptors in the myometrium *in vivo*.

Importantly, IL-1 is known to act on human amnion, chorion and myometrial cells to stimulate PGE₂ production (Pollard & Mitchell, 1996; Hertelendy *et al.* 1993; Pollard *et al.* 1993; Romero *et al.* 1989b). The mechanisms whereby IL-1 stimulates PG synthesis in myometrial cells involve stimulation of PLA₂ (Hertelendy *et al.* 1999), which aids a release of arachidonic acid from plasma membranes (Molnar *et al.* 1993), and induction of COX-2 gene and proteins (Hertelendy *et al.* 1999). In amnion and chorion cells, the stimulatory action of IL-1 on PG production involves primarily on the induction of COX-2 (Mitchell *et al.* 1993a, 1993b; Pollard *et al.* 1993). Collectively, it can be hypothesised that IL-1 may serve as a signal for parturition in the humans by acting to stimulate arachidonic acid release and induction of COX-2 with a subsequent PG formation in the human uterus.

1.7.2 IL-8 and IL-8 receptors

IL-8 is a cytokine that is capable of inducing neutrophil chemotaxis and activation. IL-8 is produced by a number of cell types including amnion, chorion, decidua, endothelial cells, endometrial cells, and fibroblasts (Trautman *et al.* 1992; Dudley *et al.* 1993; Arici *et al.* 1996; Ueno *et al.* 1996; Winkler *et al.* 1998). In addition to being a potent chemoattractant

for neutrophils, IL-8 appears to have further roles related to reproduction, as evidenced by increased levels of IL-8 in amniotic fluid throughout gestation (Saito *et al.* 1993).

IL-8 is present in amniotic fluid during early pregnancy (Srivastava *et al.* 1996), at pre-term and at term delivery, irrespective of intrauterine infection and labour status (Romero *et al.* 1991; Olah *et al.* 1996). Thus, IL-8 is also a normal constituent of amniotic fluid. Amniotic fluid from pre-term labour and delivery contains high levels of IL-8 above that of term delivery (Romero *et al.* 1991). Term parturition is associated with elevated levels of IL-8 in amniotic fluid (Romero *et al.* 1991; Saito *et al.* 1993). An increase in the level of IL-8 during labour has been reported in human amnion, chorion, decidua, placenta and lower uterine segment (Osmers *et al.* 1995; Garcia-Velasco & Arici, 1998; Winkler *et al.* 1998). Interestingly, the increased level of IL-8 in the lower uterine segment is significantly correlated with cervical dilation (Osmers *et al.* 1995; Winkler *et al.* 1998) and increased levels of collagenases from invading leukocytes (Osmers *et al.* 1995). The levels of collagenases (matrix metalloproteinase [MMP-9] and type V collagenase-gelatinase B [MMP-9]) increase dramatically with the increasing intensity of labour (Osmers *et al.* 1995). Based on these observations, IL-8 may be responsible for the migration of leukocytes and the release of these collagenases, which in turn decrease collagen content in the cervix. A decrease in cervical collagen content is known to be one of the clinical features of cervical ripening, an early event of normal labour (Ekman *et al.* 1986). Thus, IL-8 may play a crucial role in the process of cervical maturation and dilation and may be involved in the onset of labour (Osmers *et al.* 1995). Increased IL-8 levels in amniotic fluid is likely to be the result of IL-1 β and TNF α stimulation of cells in amniotic fluid. Alternatively, chorion and decidua may be another source of amniotic fluid IL-8 (Dudley *et al.* 1993). In the lower uterine segment, IL-8 may be induced by IL-1 β and fibroblasts are involved in the production of IL-8 in this part of the uterus (Winkler *et al.* 1998).

IL-8, unlike IL-1, has no direct effect on chorion, decidua and myometrial cell PGE₂ production (Dudley *et al.* 1993; el Maradny *et al.* 1996a; Todd *et al.* 1996). However, IL-8

may act as a secondary signalling mediator to potentiate the inflammatory response, thus contributing to PG production (Dudley *et al.* 1993). The result appears to be the generation of uterine contraction. IL-8 can cause a rapid rise in cytosolic free Ca^{2+} in human neutrophils (Tuschil *et al.* 1992). The relevance of this action of IL-8 in human uterus has yet to be determined. Progesterone and $\text{TGF}\beta 1$ have been found capable of influencing IL-8 production in endometrial cells in culture (Arici *et al.* 1996a, 1996b). Thus, it can be hypothesised that these agents may affect the level of IL-8 in other cell types in intrauterine tissues.

IL-8 exerts its biological activity through two receptors, designated IL-8RA and IL-8RB, which are members of the G-protein-coupled receptor family (Holmes *et al.* 1991). In the human uterus, IL-8 receptors have been demonstrated in many tissue types (el Maradny *et al.* 1996b). Using immunohistochemistry, both receptors have been found in fetal membranes, placenta, umbilical cord and myometrium samples obtained before the onset of labour. In amnion and placenta, the intensity of staining is greatest in samples collected after vaginal delivery compared to those collected before the initiation of labour. This study suggests a role for IL-8 receptors during parturition (el Maradny *et al.* 1996b). There is no data available on the regulation of IL-8 receptors in human uterus. In human neutrophils, IL-8 itself can modulate the levels of IL-8 receptors (Chuntharapai & Kim, 1995). It can be therefore postulated that IL-8 may influence the level of IL-8 receptor expression in human myometrium.

1.7.3 $\text{TGF}\beta$ and $\text{TGF}\beta$ receptors

$\text{TGF}\beta$ belongs to a family of multifunctional cytokines that are involved in the regulation of cell growth, development and differentiation, and extracellular matrix formation (Massague, 1990; Massague *et al.* 1994). There are three different isoforms of $\text{TGF}\beta$ denoted $\text{TGF}\beta 1$, $\text{TGF}\beta 2$, and $\text{TGF}\beta 3$ (Massague, 1990). $\text{TGF}\beta$ is first synthesised in cells or tissues as a large precursor molecule with an amino-terminal signal sequence, mature C-terminal peptide, and additional protein called latent $\text{TGF}\beta$ binding protein (Brand

& Schneider, 1995). This secreted TGF β is an inactive latent complex that will not bind to cell membrane receptors. Activation of this complex by proteolysis, acid or heat releases a mature 25-kDa protein, which consists of two identical disulphide-linked monomers (Barnard *et al.* 1990). This process is probably an important physiological regulator for TGF β activity (Kingsley, 1994). Three types of receptors with the molecular mass of 50-80, 85-110 and 200-400 kDa have been named as TGF β receptor type I, II and III (T β RI, T β RII and T β RIII). These receptors appear to be expressed in most mammalian cells (Massague, 1990; Kolodziejczyk & Hall, 1996).

It has been suggested that the immunosuppressive activity of amniotic fluid may be important in regulating maternal immunity during pregnancy (Shohat & Faktor, 1988). This activity of amniotic fluid has been found to correlate with the presence of amniotic fluid TGF β (Lang & Searle, 1994). A role for TGF β 1 in regulating the maternal immune response, preventing rejection of the fetus and regulating human implantation has been proposed (Kauma *et al.* 1990; Srivastava *et al.* 1996). This is because the presence of TGF β 1 in human decidua, placenta, placental membranes and amniotic fluid at first trimester (Kauma *et al.* 1990; Srivastava *et al.* 1996). In addition to TGF β , its receptors have also been demonstrated in human placenta (Mitchell *et al.* 1991; Mitchell & O'Connor-McCourt, 1992). It has been shown that the level of TGF β is increased in human amniotic fluid with pre-term labour, regardless of intrauterine infection (Mazor *et al.* 1995). Thus, it is reasonable to suggest that TGF β is a physiological cytokine, which may possess different roles at different stages of pregnancy.

In human, although TGF β and TGF β receptors have been studied at length in non-pregnant myometrium, little is known about TGF β in pregnant myometrium. Therefore, the following information is from observations at different reproductive stages in human non-pregnant myometrium. Human myometrium expresses both mRNA and proteins of all TGF β isoforms as well as T β RI-III, which are localised in non-pregnant myometrial smooth

muscle (Chegini *et al.* 1994; Dou *et al.* 1996). This finding suggests an autocrine role of TGF β in the smooth muscle of the myometrium. It has been reported that the expression of TGF β mRNA in the myometrium does not change throughout the menstrual cycle. In contrast, the levels of TGF β mRNA in human endometrium are higher during the secretory phase than the proliferative phase of the menstrual cycle, thus indicating regulation by ovarian steroids, in particular progesterone in the endometrium (Chegini *et al.* 1994; Bruner *et al.* 1995; Casslen *et al.* 1998). The role for TGF β in human myometrium is still unknown. Because of the presence of TGF β and its receptors in the myometrium and its role in cell growth and differentiation, it is possible to hypothesise that during pregnancy, TGF β influences myometrial differentiation and promote the growth of the myometrium.

Myometrial smooth muscle cells in culture contain both TGF β 1-3 and T β RI-III mRNA and proteins, and they can synthesise and release TGF β 1 into culture media (Tang *et al.* 1997). It has been found that TGF β 1 can stimulate DNA synthesis but has no effect on cell proliferation in cultured myometrial smooth muscle cells (Tang *et al.* 1997). Based on this study, TGF β 1 has been suggested to play a role in cellular hypertrophy in myometrial smooth muscle cells. This role of TGF β 1 may occur in myometrial smooth muscle *in vivo*, causing uterine enlargement during pregnancy. TGF β 1 can act on human myometrial smooth muscle cells to increase mRNA levels of parathyroid hormone-related protein (PTH-rP), immunoreactive PTH-rP secretion (Casey *et al.* 1992) and RyR2 mRNA expression (Awad *et al.* 1997). Although PTH-rP is one of several agents involved in myometrial quiescence (Morimoto *et al.* 1997), a role for PTH-rP in the initiation of parturition has been suggested (Paspaliaris *et al.* 1995). Thus, taken together, TGF β 1 may be one of the signalling agents that operates in the human myometrium during late pregnancy to regulate cell growth, promote synthesis of functional proteins and prepare the myometrium for labour (el Maradny *et al.* 1996a; Awad *et al.* 1997). TGF β 1 has been shown to have a role in the synthesis of other proteins such as endothelin-1 as seen in decidua and amnion cells during pregnancy (Kubota *et al.* 1997; McKenna *et al.* 1998).

It has been suggested that TGF β 1 acts as a gene-specific antiprogesterin (for reviews see (Casey & MacDonald, 1996, 1997). This view is based on the findings that TGF β attenuates the effect of progesterone on some genes such as endothelin and PTH-rP in endometrial stromal cells, and gap junction protein, Cx-43, in the myometrium. Possibly, the inhibition of the progesterone response by TGF β 1 can be thought as an alternative mechanism to progesterone withdrawal in human, thus contributing to the initiation of parturition.

1.8 Regulation of the levels of TGF β mRNA, TGF β secretion and activation

1.8.1 Role of sex steroid hormones

Steroid hormones can be divided into three classes; the adrenal steroids, sex steroids and vitamin D₃. It is well known that sex steroid hormones are crucial and elicit complex biological responses in the reproductive system (Koli & Keski-Oja, 1996). Cytokines are now accepted to be involved in uterine functions. An understanding of the role for cytokines in reproductive physiology, especially, during pregnancy is still in its infancy. TGF β which participates in reproductive functions as observed in animals and in humans (Tamada *et al.* 1990; Das *et al.* 1992; Chegini *et al.* 1994) has been described as a universal biological switch which is responsible for transmitting signals to the cells (Roberts & Sporn, 1993). It has been observed that steroid hormones can affect the levels of TGF β mRNA and proteins in a cell and tissue specific manner (Wakefield *et al.* 1990). Thus, in this section only the role of sex steroids on the expression and secretion of TGF β in human uterus is reviewed.

Sex steroids are known to influence the levels of TGF β mRNA and the amount of latent TGF β secretion. In endometrial stromal cells, oestradiol causes a slight increase in the level of TGF β 1 mRNA and a greater increase in TGF β 3 mRNA. Medroxy progesterone acetate (MPA) affects a very small increase in TGF β 1 mRNA level but causes a marked decrease in the level of TGF β 3 mRNA (Arici *et al.* 1996). A slight increase in TGF β 1 and

an inhibition of TGF β 2 secretion have also been demonstrated in endometrial stromal cells after oestradiol and progesterone treatment, respectively (Kanzaki *et al.* 1995). Addition of progesterone to endometrial explants enhances production of TGF β 2 mRNA (Bruner *et al.* 1995) and a combination of oestradiol and progesterone increases the level of TGF β 1 mRNA (Casslen *et al.* 1998). These findings indicate a network between endocrine and cytokine systems in human endometrial cells.

In human myometrial cell cultures, oestradiol, and in particular MPA and MPA plus oestradiol stimulate the production of total TGF β 1 (latent plus active) (Chegini *et al.* 1996). According to this study, the proportion of active TGF β 1 released into culture media is reduced by these steroids. This demonstrates an interaction between endocrines and cytokines in myometrial cells and presumably in the myometrium. Generally, TGF β is secreted from cultured cells as a latent complex, which remains biologically inactive until the latent complex is broken down. The mechanism involved in the release from latency is uncertain *in vivo* but *in vitro*, proteolytic cleavage by proteases such as plasmin may be involved (Lyons *et al.* 1990). Thus, activation of latent TGF β may be regulated by sex steroids in myometrial smooth muscle cells. This mechanism may potentially regulate TGF β 1 activity in the cells. The effect of sex steroids on other isoforms of TGF β in human myometrial cells has not yet been studied.

The mechanisms whereby sex steroid hormones affect the levels of TGF β genes and the amounts of secreted TGF β are not clearly understood. Most steroid-inducible genes contain at least one response element in the regulatory region of the gene. It has been reported that the human TGF β 3 gene contains an oestrogen response element (Yang *et al.* 1996), but there is no oestrogen response element in the TGF β 1 promoter (Kim *et al.* 1989). It has been reported that the human TGF β 1 promoter region contains activator protein-1 (AP-1) sites (Kim *et al.* 1990; Scotto *et al.* 1990) and oestrogen is known to up-regulate the expression of *c-fos* and *c-jun* genes, which are components of AP-1 (Loose-Mitchell *et al.*

1988; Weisz *et al.* 1990). Thus, stimulation of TGF β 1 by oestrogen may be regulated by the activation of transcription factors.

In other human cell types such as breast cancer cells, growth inhibition by some synthetic progestin does not involve progesterone receptor, but appears to be mediated via intracellular binding site (Colletta *et al.* 1991). Progestin can also increase the level of secreted TGF β 1 without affecting TGF β 1 mRNA (Colletta *et al.* 1991). Thus, sex steroid hormones may affect the induction of TGF β at post-transcriptional levels including efficiency of translation, post-translational processing or stability of TGF β 1 protein. The post-transcriptional mechanisms have been suggested for TGF β induction in cells that lack oestrogen receptors such as human fetal fibroblasts (Colletta *et al.* 1990).

1.8.2 Role of cAMP

There is evidence to suggest that cAMP has a strong effect on the induction of TGF β gene expression in normal trophoblasts, choriocarcinoma cells and pituitary cells (Ritvos & Eramaa, 1991; Pastorcic & Sarkar, 1997). As both cAMP and cytokines participate in the complex physiology of human gestation, it is reasonable to assume that these systems may interact with each other and play a role in co-ordinating the events of maintenance or termination of pregnancy. Unfortunately, the effects of cAMP on TGF β gene expression and TGF β secretion in the human uterus has not been well documented, except in trophoblast cells.

In normal and malignant trophoblast cells, TGF β 1 mRNA levels have been induced by the cAMP analogue, 8-bromo-cAMP, during a 48-h stimulation (Ritvos & Eramaa, 1991). It has been found that 8-bromo-cAMP has a slow effect on the activation of TGF β 1 mRNA expression, implying an indirect regulation by this agent. cAMP has been shown to induce *c-fos* and *c-jun*, which act as transcription regulators through AP-1 binding sites (Bravo *et al.* 1987; Nakamura *et al.* 1990). The TGF β 1 promoter contains no element resembling the cAMP-responsive element (CRE) sequence but contains activator protein-1 (AP-1) sites

(Kim *et al.* 1990; Scotto *et al.* 1990). Thus, cAMP may affect the expression of TGF β 1 mRNA through AP-1 binding sites present in the TGF β 1 promoter (Geiser *et al.* 1991). A direct stimulatory effect of cAMP through the AP-2 transcription factor on the TGF β 1 gene, which mediate cAMP responses can not be excluded (Ritvos & Eramaa, 1991; Pastorcic & Sarkar, 1997). Alternatively, the effect of cAMP may result from post-transcriptional control of TGF β 1 mRNA (Ritvos & Eramaa, 1991).

1.8.3 Role of cytokines

Cytokines are able to induce or inhibit synthesis and secretion of other cytokines. Thus, it has been extremely difficult to define which cytokine is responsible for a given physiological effect. A given effect may be caused by the secondarily induced cytokine rather than the primary and sometimes the full response depends on synergy between the cytokines (Clemens, 1991). It is known that cytokines have variety effects in human pregnancy and labour. TGF β 1, in particular, has been hypothesised to promote the synthesis of functional proteins in human myometrium during late pregnancy (el Maradny *et al.* 1996a; Awad *et al.* 1997). Relatively little is known about how TGF β 1 is produced in the myometrium. TGF β 1 production in myometrial smooth muscle cells may be caused by TGF β 1 itself (Tang *et al.* 1996) or by other cytokines present in the uterus such as IL-1 as reported in other cell types (Colasante *et al.* 1997). The induction of TGF β 1 expression may be regulated at several levels. These include transcription of the TGF β 1 gene through AP-1 binding sites, stability of mRNA and the efficiency of mRNA translation as described earlier.

Under natural conditions, cells have been exposed to several cytokines and other active agents at the same time, resulting in various interactions and numerous biological effects. As can be seen in section 1.8.1-1.8.3, several agents can regulate TGF β 1 expression and production. This suggests interactions between different systems for the effective operation of TGF β 1 in the cells.

1.9 Choice of cytokines

From the review above, three cytokines, TGF β 1, IL-1 and IL-8, and their receptors were chosen for study in this thesis. All TGF β isoforms have been found in the human myometrium and myometrial cells *in vitro*, with TGF β 3 being the least expressed (Chegini *et al.* 1994; Tang *et al.* 1997). It is known that myometrial smooth muscle expresses high levels of T β RII and an interaction between T β RI and T β RII is necessary for mediating the biological activities of TGF β . However, the type I receptor is used for signalling. Moreover, the intracellular Ca²⁺ release channel, RyR2, has been found to be up-regulated in human myometrial cells following TGF β 1 treatment (Awad *et al.* 1997). Thus, TGF β 1 and T β RI regulation are the principle cytokine elements investigated in this thesis.

The second cytokine studied is IL-1, along with IL-1 receptor type I. IL-1 is the major cytokine found in the amniotic fluid of pregnant women with intra-uterine infection (Mazor *et al.* 1995). IL-1 is also known to be involved in the mechanism of pre-term labour. This cytokine-induced activity is mediated exclusively via IL-1RI (Colotta *et al.* 1998). For these reasons, IL-1 and its receptor type I were chosen.

IL-8 is another cytokine chosen for investigation. This cytokine has been recognised as having a role in cervical ripening and maturation. IL-8 is present in amniotic fluid throughout pregnancy (Romero *et al.* 1991; Olah *et al.* 1996; Srivastava *et al.* 1996) and the intra-uterine levels increase during labour (Osmers *et al.* 1995; Garcia-Velasco & Arici, 1998; Winkler *et al.* 1998). There are two types of receptor mediating the IL-8 response. In the present study, IL-8 receptor type B was selected as this receptor has been reported in the myometrium and suggested for a role in human parturition. However, there is no data available on the alteration of the receptor expression in the myometrium during pregnancy and the regulation of the receptor. It is therefore of interest to examine the possible role of this cytokine in myometrial smooth muscle.

AIMS OF THE STUDY

The physiological mechanisms that initiate the onset of labour have been studied extensively in animals. It is believed that an essential part of this process involves the regulation of expression of genes encoding a cassette of proteins associated with contraction in the myometrium, the contraction associated proteins (CAPs). An alteration in the expression of CAPs has also been shown to occur in the human myometrium as term approaches and before the onset of labour. Given this perspective, the onset of labour begins when these proteins are in place. Thus, an understanding of the regulation of the expression of these proteins would be of great benefit to the management of labour. This was the overall objective of this study.

Previous work from this laboratory has suggested that the intracellular Ca^{2+} release channel RyR2 is a CAP present in the human myometrium at term. Furthermore, the cytokine $\text{TGF}\beta 1$ may be involved in the regulation of RyR2 in cultured myometrial cells. If this idea is correct, then it raises the hypotheses

1. *'that $\text{TGF}\beta 1$ and $\text{TGF}\beta$ receptors are present in the myometrium at term'.*
2. *'that expression changes with gestation'.*

The specific aim of this thesis was to examine these hypotheses. If $\text{TGF}\beta 1$ and $\text{TGF}\beta$ receptor expression change in term pregnant myometrium, this raises a further question regarding the mechanisms regulating expression. Thus, the second general aim of this thesis was to use myometrial cells *in vitro* to examine the effects of cytokines such as $\text{TGF}\beta 1$, IL-1 and IL-8, sex steroid hormones and cAMP on the expression of $\text{TGF}\beta 1$ and $\text{TGF}\beta$ receptor type I.

Chapter 2

METHODS

2.1 Myometrial collection and ethical approval

2.1.1 Myometrial collection

Samples of myometrium were taken from the uterine segment from pre-menopausal patients undergoing hysterectomy or caesarean section at Royal Victoria Infirmary, Newcastle upon Tyne. Control samples from non-pregnant women of reproductive ages ($n = 30$) were obtained from patients undergoing hysterectomy due to benign gynaecological disorders such as benign tumours. In this group, myometrial samples were taken from the anterior wall of the lower uterine body, immediately following hysterectomy. In the case of caesarean section, myometrial samples were obtained from the upper margin of the lower uterine segment. Samples of pregnant myometrium were collected from three groups of women as follows:

1. Pre-term women (31-35 weeks gestation, $n = 5$) without intrauterine infections and labour pain: myometrial samples were obtained at caesarean section due to fetal distress or small for gestational ages. This group was called the pre-term non-labouring group.
2. Term pregnant women (38-40 weeks gestation, $n = 22$) who had not entered labour: myometrium was obtained from patients with no complications at elective caesarean section. The indications for caesarean section were either previous section or cephalo-pelvic disproportion. This group was called the term pregnant non-labouring group.
3. Term pregnant women (38-40 weeks gestation, $n = 20$) who were in regular uterine contractions with the cervix 6-10 cm dilated. The diagnosis for this group was fetal distress or failure to progress. In this group, amniotic membranes were intact with no history or presence of infection at the time of caesarean section. Patients who had augmentation of labour with oxytocin were included in this group. This group was called the spontaneous labouring group.

2.1.2 Ethical approval

Patients were consented for myometrial biopsies on the day before their operations in the case of hysterectomies and planned elective caesarean sections or immediately prior to the operations in the case of emergency caesarean sections. The present study had ethical approval from the Newcastle Area Health Authority.

2.1.3 Removal of myometrium from the uterus

Samples of 1x1x2 cm were removed from the lower anterior uterine segment and placed in collection medium (Medium 199 supplemented with 2 mM glutamine, 500 units penicillin and 100 µg/ml streptomycin). The myometrium was excised about 5 mm away from the decidua, serosal layer, or tumour. Since it has been reported that these tissues may contain cytokines or cytokine receptors, it was important to remove all such tissues from the myometrium. Great care was taken to obtain pure myometrial samples. Samples were washed in phosphate-buffered saline (PBS) and cut into small pieces before being rapidly frozen in liquid nitrogen. Samples were kept at -70 °C until used for preparing myometrial lysates (see section 2.2). Samples of myometrium were also used for immunohistochemistry using a paraffin-embedding technique (see section 2.3). In this case, the samples were kept in formaldehyde. In addition, non-pregnant myometrium samples were used to prepare cultured cells. These samples were diced into small pieces and myometrial cells were isolated using enzymatic digestions (see section 2.4.1).

2.2 Preparation of human myometrial lysate

Small pieces of individual frozen myometrium from each group were weighed and then thawed in 3 volumes of cold homogenising buffer containing 25 mM Tris base, pH 7.6, 0.25 M sucrose and 1 mM EDTA (Europe Finner *et al.* 1993). The myometrium was further disrupted by mechanical homogenisation on ice. The homogenates were then centrifuged at 1,500 g for 30 min at 4 °C. The supernatants were removed and re-centrifuged to obtain clear lysates. Protease inhibitors: 5 µg/ml of pepstatin A, 5 µg/ml

leupeptin and 5 µg/ml aprotinin were added into the lysates to prevent protein degradation. The protein content in lysates was measured by the method of Bradford (1976) (see section 2.6). Myometrial lysates were aliquoted and kept frozen at -70°C until used.

2.3 Immunohistochemistry

2.3.1 Tissue section determination of TGFβ receptors in human myometrial smooth muscle

Non-pregnant, term pregnant non-labouring and term spontaneous labouring myometrium were washed with $\text{Ca}^{2+}/\text{Mg}^{2+}$ free PBS and the serosal layer removed. Samples of myometrium were fixed in 4 % formaldehyde buffered to prevent structural changes in the tissues. The fixed tissues were dehydrated by placing in absolute alcohol. This step was essential because in this technique all the water in tissues is replaced by paraffin wax so that the tissue blocks can be sliced easily. Once absolute alcohol had replaced water in the tissues, the tissue blocks were passed through xylene three times, with the result that xylene replaced the alcohol in the tissues. Warm paraffin wax, which is soluble in xylene was introduced to the tissue blocks until the tissues become saturated with paraffin. Tissue sections (5 µm) were then cut with a microtome, placed onto glass slides for use.

2.3.2 Indirect immunostaining method

Before sections were stained, paraffin was removed by xylene. The slides were then hydrated through the decreasing concentrations of alcohol (100 %, 95 % and 50 %) and finally tap water for 2 min each stage to remove xylene. The endogenous peroxidase activity was blocked by incubating the slides with 3 % hydrogen peroxide for 10 min and subsequently washing for 2 min with tap water and PBS, pH 7.3. The slides were incubated for 30 min with blocking serum derived from the same species in which the secondary antibody was made. In this experiment, the blocking serum was goat serum. Excess serum was removed and the slides were now ready for staining with primary antibodies.

The slides were incubated with rabbit antibodies to TGF β receptor types I, II and III from Insight Biotechnology at a concentration of 3 μ g/ml diluted in PBS with 1 % goat serum for 2-3 h in a humidified chamber. The slides were washed three times for 5 min each time with PBS and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:100) from Dako for 45 min. The slides were washed twice with PBS and the bound antibodies visualised by incubating the slides with freshly prepared diaminobenzidine (DAB) for 5 min or until a brown stain was seen. The slides were washed for 2 min with tap water, counter-stained with hematoxylin for 1 min and rinsed immediately with running tap water. The stained slides were dehydrated through 95 % alcohol, absolute alcohol, and then through xylene for 1 min each stage. The excess xylene was wiped off and 1 or 2 drops of xylene-based mounting medium added, covered with glass coverslips, observed under light microscope and photographed. A negative control was processed in an identical manner, but in the absence of primary antibody. All procedures were carried out at room temperature.

2.4 Human myometrial cell culture techniques

2.4.1 Isolation of human myometrial smooth muscle cells

Myometrial smooth muscle cells were prepared by enzymatic digestion using a technique modified from the procedures described by Morgan *et al* (1993). Samples of non-pregnant myometrium in sterile collection medium were dissected from any adherent endometrium and washed twice with Ca²⁺/Mg²⁺ free PBS. The cleaned myometrial tissue samples were sliced into small pieces and placed in sterile tubes. 5 ml of digestion enzymes composed of 5 mg collagenase, 0.1 mg DNase, 1 mg trypsin inhibitor, 1 mg elastase and 1 mg bovine serum albumin fraction V in Ca²⁺/Mg²⁺ free PBS were added into each tube. The myometrial slices were digested at 37 °C with shaking for 2 h. Enzyme solutions containing cells were removed to sterile centrifuge tubes and the connective tissues were left behind. 5 ml complete medium [collection medium supplemented with 10 % fetal calf serum (FCS)] was added to the tubes to stop enzyme action and to prevent cell damage. The tubes were centrifuged for 10 min at 400 g and cell pellets re-suspended in 10 ml complete

medium. The cell suspensions were transferred to sterile 75 cm² flasks and placed in a 5 % CO₂ humidifying incubator at 37 °C. The cells were left in the flasks for approximately 24 h to allow cells to attach. Once cells were attached the medium was changed and the new complete medium was replaced twice a week. Normally, confluence was achieved within 10 days in culture. This may depend on the yield of cells obtained from digestion and cells plating density.

2.4.2 Cell passage

Confluent cells were washed twice with 15 ml Ca²⁺/Mg²⁺ free PBS to remove any FCS and Ca²⁺, which would interfere with the action of the enzyme trypsin. Cells in 75 cm² flasks were incubated at 37 °C with 5 ml of 0.5 g/L trypsin containing 0.2 g/L EDTA for 10 min. The flasks were gently shaken to detach cells from the base of the flasks. If cells were seen rounded up and phase bright in the enzyme solution, 5 ml of complete medium was added to terminate enzyme action. The cell suspensions were transferred to centrifuge tubes, centrifuged for 10 min at 400 g and cell pellets re-suspended in 10 ml complete medium. The cells in complete medium were passed gently through a pipette approximately 5 times to dissociate the clumps of cells. After cell number estimation (see section 2.4.3), 1 x 10⁶ cells were diluted in 15 ml culture medium and plated into 75 cm² flasks for further culture. Cells required for experiments were seeded either onto 12 well tissue culture plates (5 x 10⁴ cells/well) or 25 cm² flasks (2 x 10⁵ cells). Cell counts were made each day for the period of 10 days. Seeding at these numbers of cells would allow approximately 7 days for the cells to achieve confluence. Confluent cells from passage one to passage six were used in all experiments.

2.4.3 Estimation of cell numbers

The concentration of cells was determined, where necessary, by counting the cells in a haemocytometer (Improved Neubauer). Cells were trypsinised and 20 µl of cell suspension was counted in duplicate. For routine subcultures, 100-300 cells per mm² were counted and

for experiments, 500 cells per mm² were counted. The two counts were averaged and the concentration of cells were calculated from the following formula

$$c = n/v$$

Where c = cell concentration (cells/ml), n = number of cells count, and v = volume counted (ml). For the Improved Neubauer slide, the central 1 mm² was used and the depth of the chamber is 0.1 mm, thus, $v = 0.1 \text{ mm}^3$ or 10^{-4} ml. The formula becomes

$$c = n \times 10^4$$

2.4.4 Verification of myometrial smooth muscle cell purity

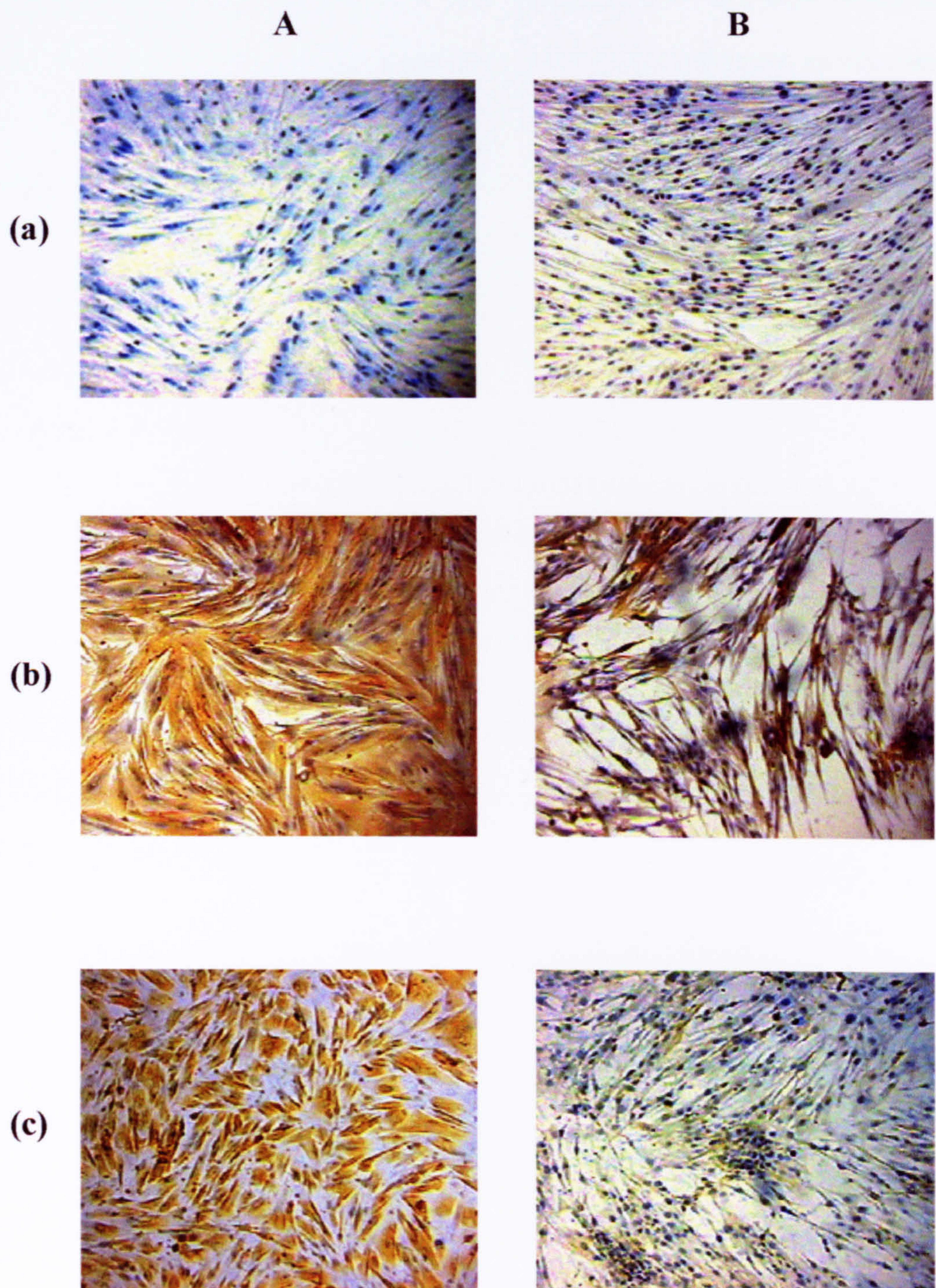
In order to ensure that cells were myometrial smooth muscle, cells were verified by immunoperoxidase staining with monoclonal antibodies to smooth muscle α -actin and calponin from Dako. Confluent cells grown on sterile glass coverslips were washed twice with PBS. The cells were then fixed with 95 % methanol for 10 min. After fixing, the coverslips were rinsed briefly with PBS. The cells were permeabilised with 0.05 % saponin in de-ionised water for 15 min and washed twice with PBS. The cells were then incubated for 10 min in 3 % hydrogen peroxide to quench endogenous peroxidase activity. Subsequently the cells were washed in running tap water and PBS for 2 min each. The cells were incubated in blocking serum derived from the same species in which the secondary antibody was made for 30 min to suppress non-specific binding of IgG. Excess blocking serum was removed before incubation with primary antibodies. The subsequent incubations were carried out in a humidified chamber at room temperature. The coverslips were covered with 100 μ l of mouse anti-smooth muscle α -actin and calponin antibodies at 1:20 and 1:40 dilutions, respectively and incubated for 90 min at room temperature. The coverslips were washed with three changes of PBS. After the washing steps, cells were incubated for 45 min with HRP-conjugated goat anti-mouse-IgG (1:100). The cells were then washed twice for 5 min each time with PBS, incubated in freshly prepared diaminobenzidine (DAB) solution for 5 min or until brown staining was visible and subsequently washed for 2 min with tap water.

The cells were counter-stained with hematoxylin, rinsed in running water and mounted in aqueous mounting medium. Negative control myometrial cultured cells were processed in an identical manner, but the primary antibody was replaced with mouse IgG. Specificity of these two antibodies to smooth muscle was checked by incubation of fibroblast cells derived from human prostate with these antibodies in the same manner. The immunostaining was observed by light microscopy and photographed using a Nikon FG-20 SLR camera loaded with Fujichrome tungsten balanced film, attached to the front port of the microscope. Results show that only a few fibroblast cells are positively stained with these antibodies. Additionally, greater than 95 % of myometrial cells are positively stained, suggesting that they are of smooth muscle origin. Figure 2.1 shows photomicrographs of confluent myometrial cells and fibroblast cells stained with α -actin and calponin antibodies.

The cells were counter-stained with hematoxylin, rinsed in running water and mounted in aqueous mounting medium. Negative control of non-specific staining cells were processed in an identical manner, but the primary antibody was replaced with mouse IgG. Specificity of these two antibodies to smooth muscle was checked by incubation of fibroblast cells derived from human prostate with these antibodies in the same manner. The immunostaining was observed by light microscopy and photographed using a Nikon FG-20 SLR camera loaded with Fujisone tungsten balanced film attached to the front port of the microscope. Results show that only a few fibroblast cells are positively stained with these antibodies. Additionally, greater than 95% of non-specific cells are positively stained, suggesting that they are of smooth muscle origin. Figure 2.1 shows photomicrographs of confocal

Figure 2.1 Immunoperoxidase staining for α -actin and calponin in monolayer cultures of myometrial smooth muscle cells and fibroblast cells.

The figure shows photomicrographs of culture myometrial smooth muscle cells, column A and fibroblast cells, column B. Both cell types were stained with mouse IgG (a), α -actin (b) and calponin (c).



2.5 Treatment of cultured myometrial smooth muscle cells with Cytokines, steroid hormones, antibodies to cytokines and forskolin

In these investigations, myometrial cells were treated with IL-1 at a dose chosen to be maximal for prostaglandin stimulation in human myometrial cells (10 ng/ml) (el Maradny *et al.* 1996a; Todd *et al.* 1996). This concentration has also been reported to exist in the amniotic fluid of women with infection-associated pre-term labour (Romero *et al.* 1989a). The dose of IL-8 (150 ng/ml) was chosen from a range that stimulates transient mobilisation of Ca^{2+} in human keratinocytes (Tuschil *et al.* 1992). For the stimulation of cells with TGF β 1, 1 ng/ml was used as the RyR2 mRNA in the human myometrial cells can be up-regulated at this concentration (Awad *et al.* 1997).

In this study the myometrial cells *in vitro* were maintained in the medium containing serum. All of the experiments were done from this base line condition. It is well known that serum can contain many factors, which may affect the responsiveness of cells. However, in this thesis we chose to work under conditions where the cells were viable and healthy (i.e. in serum containing medium). It must be borne in mind that the data obtain are therefore specific for serum-treated cells.

2.5.1 IL-1 and IL-8 treatment and determination of TGF β 1 and T β RI expression

Myometrial cells were cultured in 12 well plates until confluence. The cells in complete medium were incubated for 24 h with or without IL-1 (10 ng/ml) or IL-8 (150 ng/ml). The bathing culture medium was collected at the end of each treatment and centrifuged at 1000 g for 10 min at 4 °C. Supernatants were kept at -70 °C for subsequent determination of TGF β 1 levels using enzyme-linked immunosorbent assay (ELISA) as described in section 2.10. The cells were washed with Ca^{2+} /Mg $^{2+}$ free PBS and trypsinised. The trypsinised cells in complete culture medium were centrifuged at 400 g for 5 min. The cell pellets were washed, re-suspended in 40 μ l homogenising buffer containing protease inhibitors (see

section 2.2) and passed through a pipette five times. The protein content was determined for each sample by the Bradford assay (1976). The level of T β RI in cells was determined using electrophoresis and immunoblotting (see section 2.7-2.8).

2.5.2 TGF β 1 and IL-8 treatment and determination of T β RI and IL-8RB

Confluent myometrial cells in 12 well plates were treated for 24 h with or without TGF β 1 (1 ng/ml) and IL-8 (150 ng/ml) in complete culture medium. After treatments, the cells were processed as described in section 2.5.1 and the levels of T β RI and IL-8RB determined using Western immunoblotting analysis (see section 2.7-2.8).

2.5.3 Sex steroid hormones treatment and determination of TGF β 1 and T β RI

Confluent myometrial cells in 12 well plates were treated for 24 h with 17 β -estradiol (10 and 100 nM), progesterone (100 and 200 nM) or in combination with IL-1 (10 ng/ml) or IL-8 (150 ng/ml). Control cells received complete medium alone. At the end of each treatment the bathing culture medium was collected and centrifuged at 1000 g for 10 min at 4 °C. Supernatants were kept at -70 °C for subsequent determination of TGF β 1 levels using ELISA as described in section 2.10. The cells were processed as described in section 2.5.1 and the levels of T β RI in cells determined using Western immunoblotting analysis (see section 2.7-2.8).

2.5.4 Cytokines and cytokines neutralising antibodies treatment and determination of T β RI

Confluent myometrial cells in 25 cm² flasks were pre-incubated for 3 h with TGF β 1 neutralising antibody (TGF β 1_(NA)) or IL-1 neutralising antibody (IL-1_(NA)) from R&D systems at concentrations of 3 μ g/ml or 0.5 μ g/ml, respectively. Following pre-treatment with TGF β 1_(NA), the cells in complete culture medium were incubated for 24 h with IL-1 (1 ng/ml) or IL-8 (150 ng/ml) in the presence of neutralising antibody. For the cells pre-treated with IL-1_(NA), the cells were stimulated with IL-8 (150 ng/ml) for 24 h. Cells were also treated with IL-1 and IL-8 in parallel to these treatments. Control cells were incubated

in complete culture medium alone. The cells were harvested and the levels of T β RI expression determined using Western immunoblotting analysis (see section 2.7-2.8).

2.5.5 Cytokines and cytokines neutralising antibodies treatment and determination of cyclooxygenase-2 (COX-2)

Confluent myometrial cells in 25 cm² flasks were pre-incubated for 3 h with TGF β 1 neutralising antibody (TGF β 1_(NA)) or IL-1 neutralising antibody (IL-1_(NA)) at concentrations of 3 μ g/ml or 0.5 μ g/ml, respectively. Following pre-treatment with TGF β 1_(NA), the cells in complete culture medium were incubated for 24 h with IL-1 (1 ng/ml) in the presence of neutralising antibody. For the cells pre-treated with IL-1_(NA), the cells were stimulated for 24 h with TGF β 1 (1 ng/ml). The cells were also treated with TGF β 1 and IL-1 in parallel to these treatments. Control cells were incubated in serum culture medium alone. The level of COX-2 expression was determined at the end of treatment using Western immunoblotting analysis (see section 2.7-2.8).

2.5.6 Forskolin treatment and determination of TGF β 1, T β RI, c-Fos and c-Jun

Confluent myometrial cells in 12 well plates were incubated for 24 h, 48 h and 72 h with forskolin (5 μ M) in the presence of serum. The control cells were incubated in complete culture medium alone. At the end of treatment, culture medium was removed and centrifuged at 1000 g for 10 min at 4 °C. The levels of TGF β 1 in cultured supernatants were measured using TGF β 1 ELISA (see section 2.10). The cells were washed with PBS and lysed with lysis buffer supplied in cAMP assay kit (see Appendix 5). Intracellular cAMP levels were measured using a cAMP enzyme immunoassay (see section 2.11). In addition, confluent cells in 25 cm² flasks were incubated for 45 min, 2 h, 24 h, 48 h and 72 h with similar treatment. At the end of each incubation period, the cells were harvested and processed as described in section 2.5.1. Levels of T β RI, c-Fos and c-Jun were analysed using Western immunoblotting (see section 2.7-2.8).

2.5.7 TGFβ1 treatment and determination of the G protein sub-units (Gα_s and Gα_{i1,2})

Confluent myometrial cells in 12 well plates were treated for 24 h with TGFβ1 (1 ng/ml). After treatments, the cells were processed as described in section 2.5.1 and the levels of Gα_s and Gα_{i1,2} in the cells determined using Western immunoblotting (see section 2.7-2.8).

2.6 Total protein assay

Protein concentration was assayed using a Bio-Rad Protein assay kit based on the method of Bradford (1976). This method is based on the observation that Comassie Brilliant Blue G-250 exists in two different colours, red and blue. When binding to protein occurs, the maximum absorption of the dye shifts from 465 to 595 nm as the red form is converted to the blue form. The differential colour change of the dye because of various amounts of protein is determined at 595 nm.

To assay protein concentration, several dilutions of bovine albumin (200-1400 µg/ml) were prepared. A standard curve was carried out each time the assay was performed. Samples, to be assayed, were diluted in distilled water. The concentrated dye reagent was diluted 1:4 with distilled water.

Protein standards and appropriately diluted samples in a volume of 20 µl were added into dry test tubes. 20 µl of distilled water was also placed in a test tube as a blank. Diluted dye reagent (1 ml) was added to each tube and the contents mixed gently. After incubation between 5-60 min at room temperature, the absorbance at 595 nm was measured against a reagent blank. A standard curve was plotted and protein concentrations in the samples calculated and multiplied by the appropriate dilution factor to give µg/ml. All samples were analysed in duplicate.

2.7 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting

The solutions for SDS-PAGE including formulation of stacking gels, separating gels and electrophoresis buffer are shown in Appendix 3.

2.7.1 Protein sample preparation and gel electrophoresis

7.5 % or 12.5 % separating mini-gel was made and overlaid with isopropyl alcohol to give a flat surface. Once the separating gel had polymerised, the isopropyl overlay was removed and the separating gel surface rinsed with distilled water. 5 % stacking gel was layered on top of the separating gel, and a comb with the required number of wells was carefully inserted. When polymerisation had occurred, the comb was removed, leaving behind the sample wells. The wells were then covered with electrophoresis running buffer.

Protein samples from myometrial tissue lysates or cultured myometrial smooth muscle cells (40-50 µg) were prepared. Laemmli buffer (2x) containing 0.125 M Tris-HCl, pH 6.8, 4 % SDS, 10 % 2-mercaptoethanol, 20 % glycerol and 0.004 % bromophenol blue tracking dye was added to the samples at 1:1 (V/V) dilution. Samples were mixed, heated for at least 5 min at 95 °C and collected by a brief centrifugation. Samples and standard markers were loaded into the wells and subjected to electrophoresis in a mini-PROTEAN II cell according to the method of Laemmli (1970). The gel was run at 30-35 mA until the tracking dyes reached the base of the gel. The gel was removed and proteins from the separating gel transferred onto nitro-cellulose membranes using an electroblotting method. Semi-dry blotting was found to be less efficient, therefore, the wet blotting with a transfer buffer containing methanol (Towbin *et al.* 1979) was the system used in preference for transferring proteins in this thesis.

2.7.2 Western Blotting: wet blotting system

Approximately 5 min prior to finishing the electrophoresis, the nitro-cellulose membrane (0.45 µm) purchased from Schlicher & Schuell and six pieces of Whatman 3MM

filter papers were cut to the size of the separating gel and pre-soaked in freshly prepared transfer buffer (see Appendix 3). This prevents air bubbles becoming trapped in the membranes. One of the corners of membrane was labelled to orientate the samples. A clean tray containing transfer buffer was prepared and the cassette to hold the gels was placed in the tray. A sponge pad was centred on the black side of the cassette (cathode) and air bubbles were removed from the sponge. Three pieces of Whatman filter papers were placed on top of the sponge pad. Air bubbles were removed by rolling a pipette over the papers. After samples had undergone electrophoresis, the stacking gel and unused gel were trimmed and the separating gel soaked in transfer buffer for 15 min to remove electrophoresis buffer salts and reduce swelling of the gel. The nitro-cellulose membrane was then placed smoothly on the gel (label toward gel). The gel together with membrane were carefully lifted up and placed on the filter papers in a cassette. Air bubbles were removed by gently squeezing. Three more filter papers were placed on the membrane followed by another sponge pad. The bubbles were removed by carefully rubbing or pressing. The other side of the cassette was covered and the cassette locked. The cassette and the cooling system supplied with the apparatus, in this case, frozen water in a container were placed into a chamber. The chamber was then filled with cold transfer buffer, and the system (Bio-Rad wet blotting) run at 100 V for 60 min. After transfer, the gel was stained with Coomassie blue and the membrane was stained with Ponceau-S to check the efficiency of the transfer. Ponceau-S was washed away with Tris-buffered saline (TBS) or PBS. The immobilised proteins on the membranes were then detected using immunoblotting analysis. The membranes can be stored at this stage by air-drying and kept at -20 °C.

2.8 Immunoblotting and enhanced chemiluminescence system (ECL)

After transfer, the membrane was blocked overnight in TBS (TBS: 10 mM Tris-HCl, 150 mM NaCl, pH 8) containing 5 % non-fat dried milk. The membrane was rinsed with two changes of TBST (TBS and 0.05 % Tween-20). The membrane was incubated at room temperature with an appropriate primary antibody diluted in TBST containing 1 % non-fat

dried milk for the indicated time as shown in Table 1. Following three 15-min washings with TBS, the specific secondary antibody conjugated to horseradish peroxidase in TBST was applied to the membrane at a dilution of 1:2500 for the time indicated in Table 1. The membrane was then washed three times with TBST. All procedures were performed on a shaking platform at room temperature (20-22 °C). Following antibody incubation, the excess buffer from the washed membranes was drained. The enhanced chemiluminescence reagents from Amersham were mixed in 1:1 dilution and added to cover the membranes. The incubation time was exactly 1 min. The excess reagents were drained from the membrane and the membrane was wrapped in cling film. All air bubbles were removed. The membrane was placed in the film cassette, protein side up. The following steps were carried out in a photographic dark room illuminated by the red light. X-ray film was placed on the membrane. The cassette was closed and film was exposed for 15 sec. The first film was developed immediately to estimate the exposure time for the next films. The molecular mass of protein was estimated by comparing the immunoreactive band to the standard makers on the membrane from the same blot. The schematic representation of Western immunoblotting is shown in Figure 2.2.

Table 1. The concentrations and incubation times of primary and secondary antibodies used for immunoblotting analysis

Primary antibody	Concentration and incubation time	Secondary antibody	Dilution and incubation time
TGFβ receptor type I (TβRI)	0.5 µg/ml, 2 h and 30 min	Goat anti-rabbit	1:2500, 1 h
TGFβ receptor type II (TβRII)	0.5 µg/ml, 2 h and 30 min	Goat anti-rabbit	1:2500, 1 h
TGFβ receptor type III (TβRIII)	0.5 µg/ml, 2 h and 30 min	Donkey anti-goat	1:2500, 45 min
Interleukin-1 receptor type I (IL-1RI)	1 µg/ml, 2 h and 30 min	Goat anti-rabbit	1:2500, 1 h

Interleukin-8 receptor type B (IL-8RB)	0.5 µg/ml, 2 h and 30 min	Goat anti-rabbit	1:2500, 1 h
Cyclo-oxygenase-2 (COX-2)	0.5 µg/ml, 2 h and 30 min	Donkey anti-goat	1:2500, 45 min
c-Fos	1 µg/ml, 2 h	Goat anti-rabbit	1:2500, 1 h
c-Jun	0.5 µg/ml, 2 h	Goat anti-rabbit	1:2500, 1 h
Gβ	1:1000	Goat anti-rabbit	1:2500, 1 h
Gα _s	1:1000	Goat anti-rabbit	1:2500, 1 h
Gα _i	1:1000	Goat anti-rabbit	1:2500, 1 h

The primary antibodies except G-proteins sub-units and HRP-conjugated donkey anti-goat were purchased from Insight Biotechnology Ltd. G-protein sub-units were obtained from NEN Life Science products, Inc. HRP-conjugated goat anti-rabbit or mouse are purchased from Dako Ltd.

2.9 Quantification of immunoreactive bands

Band intensities were evaluated by densitometric scanning using a GS-690 densitometer and analysed with Multianalyst software from Bio-Rad Laboratory. All assay conditions such as reagent concentrations and incubation time were controlled in order to standardise procedures. In spite of standardisation, differences between blots occurred. Therefore, the intensity values obtained from non-pregnant myometrium or control cells were set at 100 %. It is possible to obtain an estimate of the variability of the expression in the non-pregnant myometrium or control cells by comparing these samples on the same gel. This variability is illustrated by the error bar on the 100 % level for each of the experiments. Data were obtained under conditions where a linear relationship existed between the amounts of protein loaded and the intensity of the immunoreactive signals as shown in Figure 2.3. Linearity between amounts of protein and immunoreactivity was demonstrated for TβRI, IL1-RI and IL-8RB. This relationship was approximately linear up to 90 µg of protein per

lane. Data in each study represents the mean values of at least four determinations from four different samples.

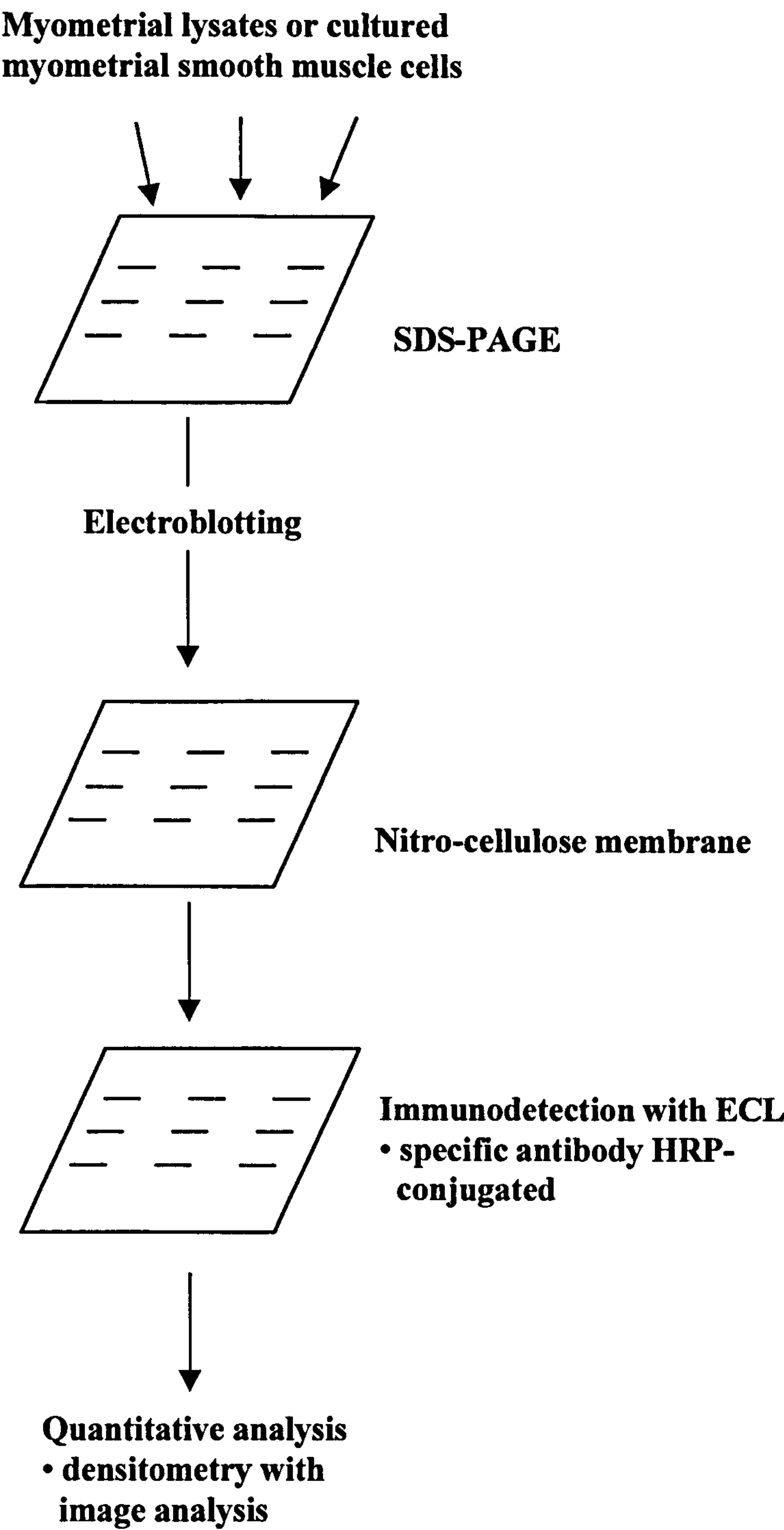


Figure 2.2 Schematic representation of Western immunoblot analysis

Western blot analysis of myometrial tissue lysates



Figure 2.3

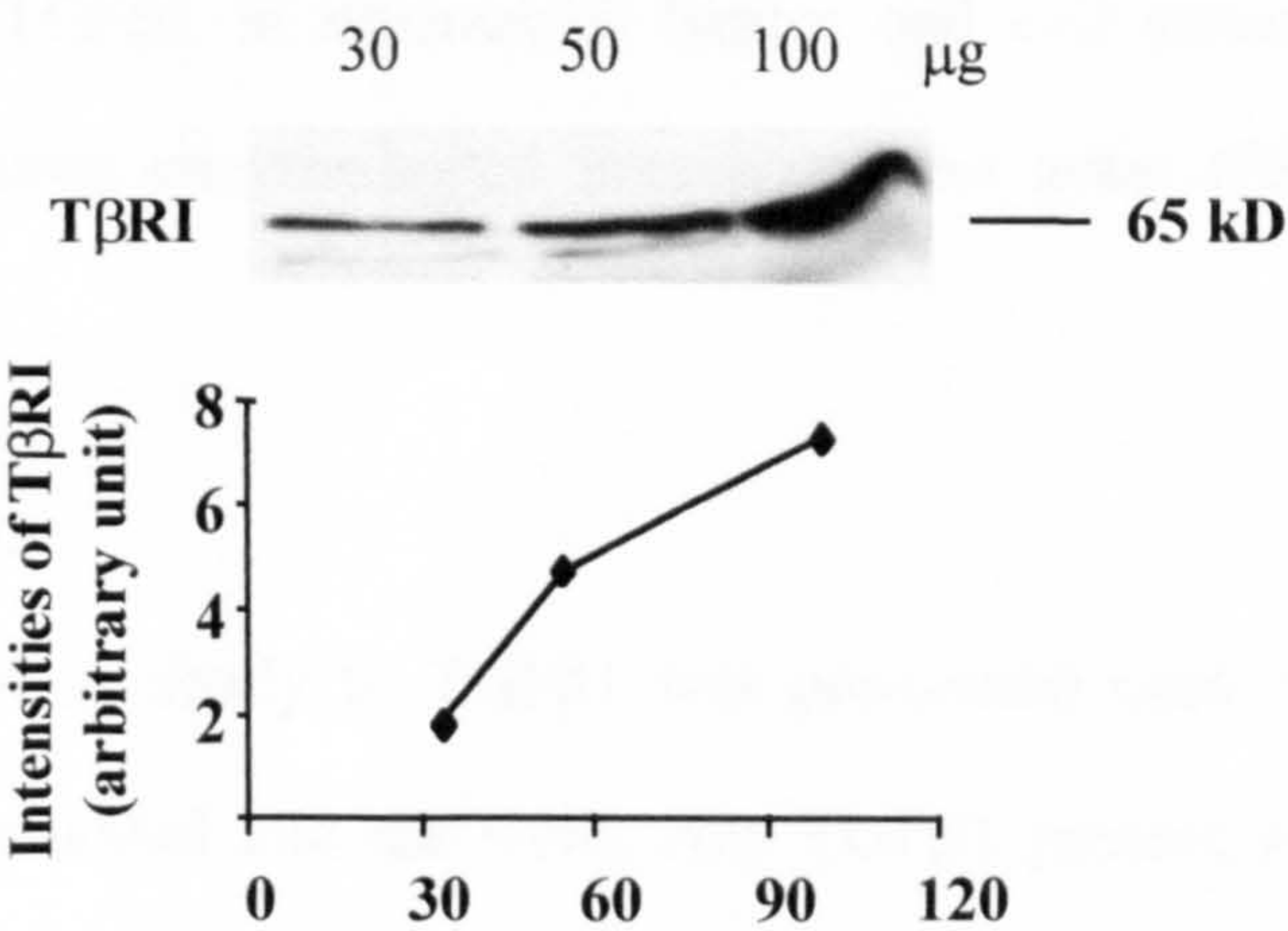
Figure 2.3 Linearity between the amounts of protein and intensities of immunoreactive signal for T β RI, IL-1 RI and IL-8RB in myometrial tissue lysates.

- A. Top panel: Western blot illustrating the expression of T β RI prepared from non-pregnant myometrium. Bottom panel: the intensity level of T β RI against different amounts of protein.
- B. Top panel: Western blot illustrating the expression of IL-1RI prepared from non-pregnant myometrium. Bottom panel: the intensity level of IL-1RI against different amounts of protein.
- C. Top panel: Western blot illustrating the expression of IL-8RB prepared from non-pregnant myometrium. Bottom panel: the intensity level of IL-8RB against different amounts of protein.

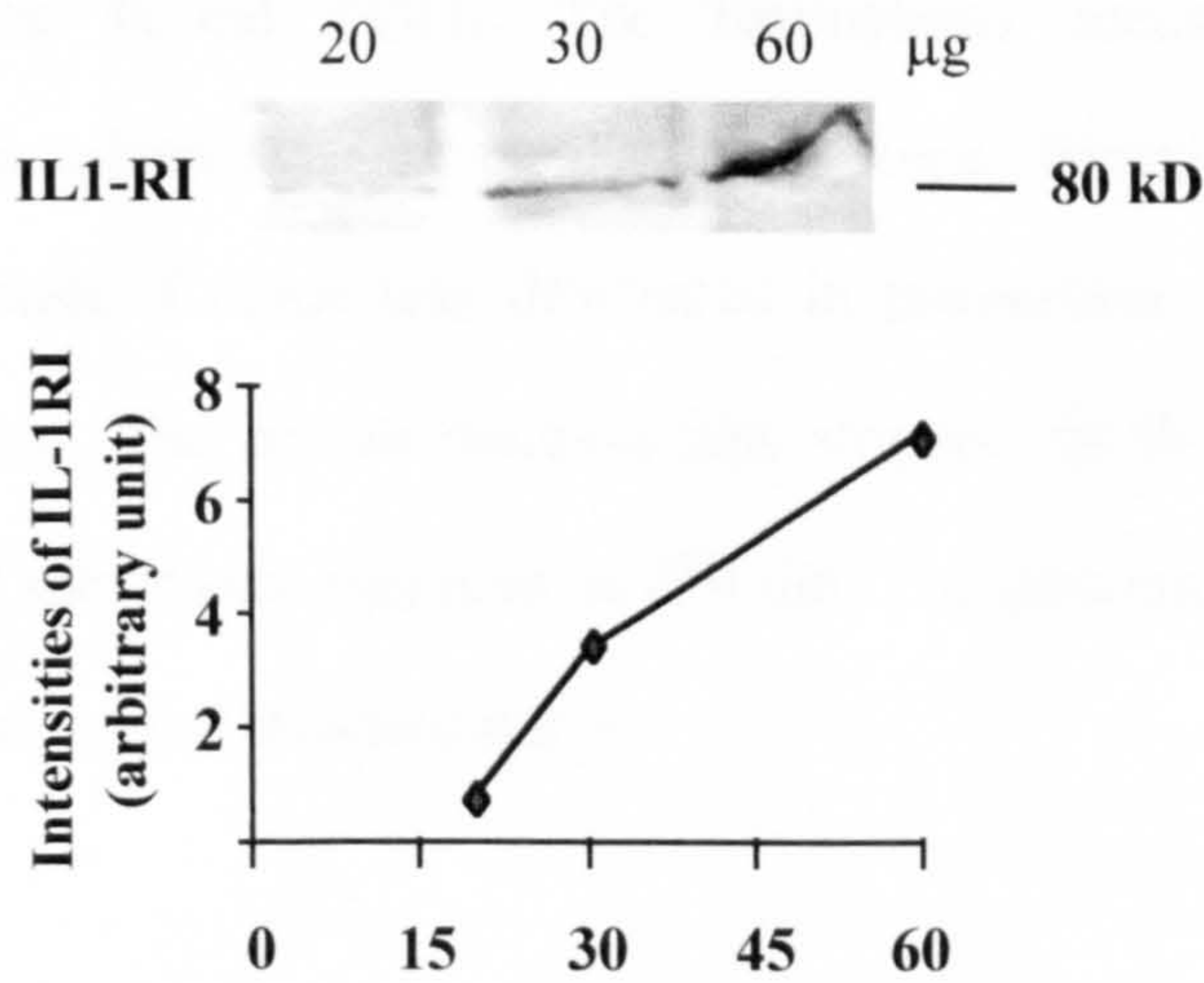
Western blot analysis of myometrial tissue lysates



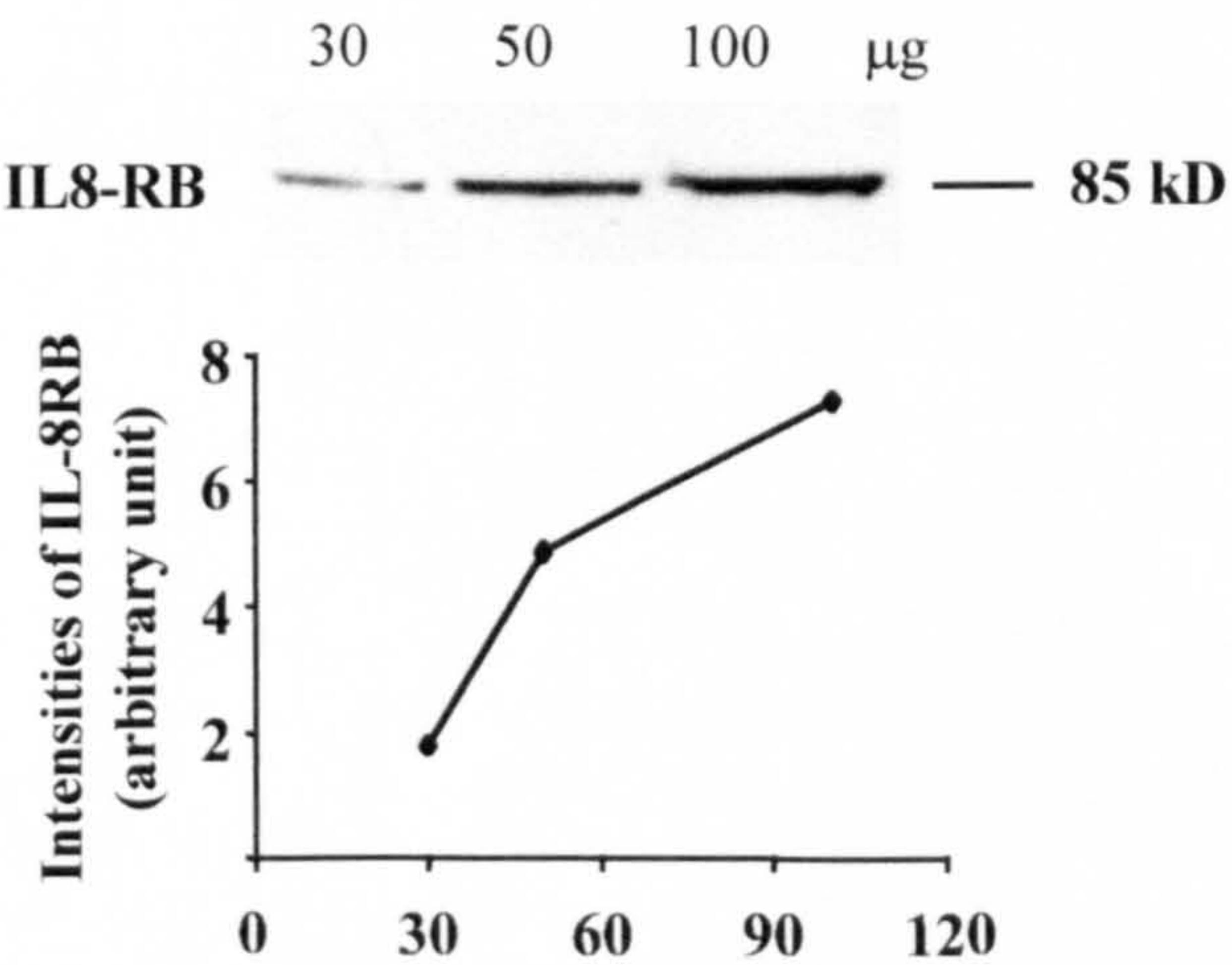
A.



B.



C.



Protein concentration (μg)

2.10 TGF β 1 immunoassay

Steady-state levels of TGF β 1 in myometrial lysates and cell culture medium after treatment were measured using enzyme-linked immunosorbent assay (ELISA) kits from Amersham.

2.10.1 Principle of the assay

As shown in Figure 2.4, antibody to TGF β 1 was pre-coated onto micro-titre wells. Standards and samples were added into the wells. Any TGF β 1 present was bound to the wells and other unbound components were washed away. Antibody to TGF β 1 was added to the wells to sandwich the bound TGF β . The biotinylated second antibody and streptavidin/peroxidase were added after several washing steps. Peroxidase activity was determined by adding substrate. Colour was developed in proportion to the amount of TGF β bound in the first step. The colour reaction was stopped by the addition of acid solution and the intensity of the colour was read at 450 nm. The concentrations of TGF β 1 in the samples were then read from a standard curve.

2.10.3 Sample preparation

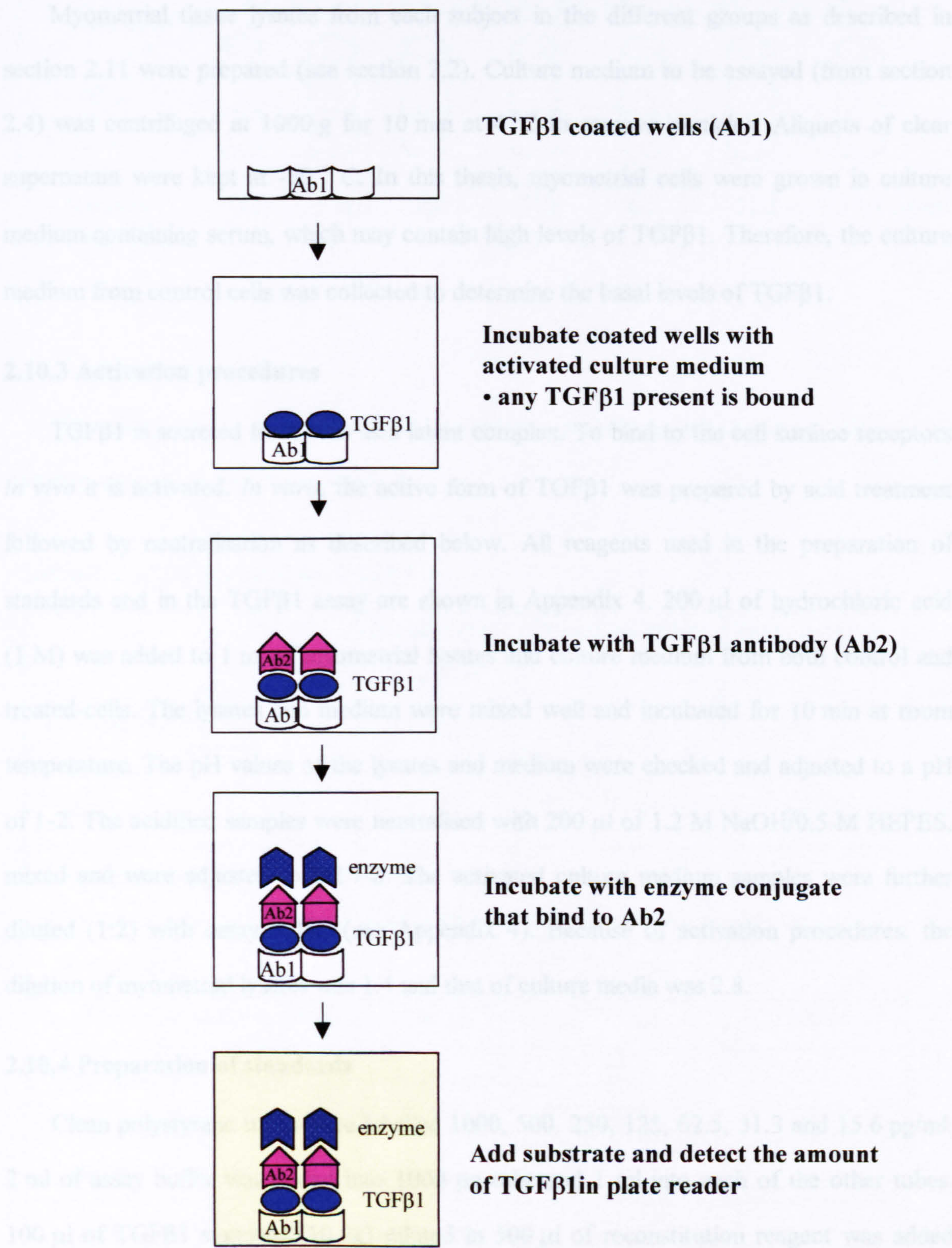


Figure 2.4 Schematic representation of a sandwich ELISA

2.10.2 Sample preparation

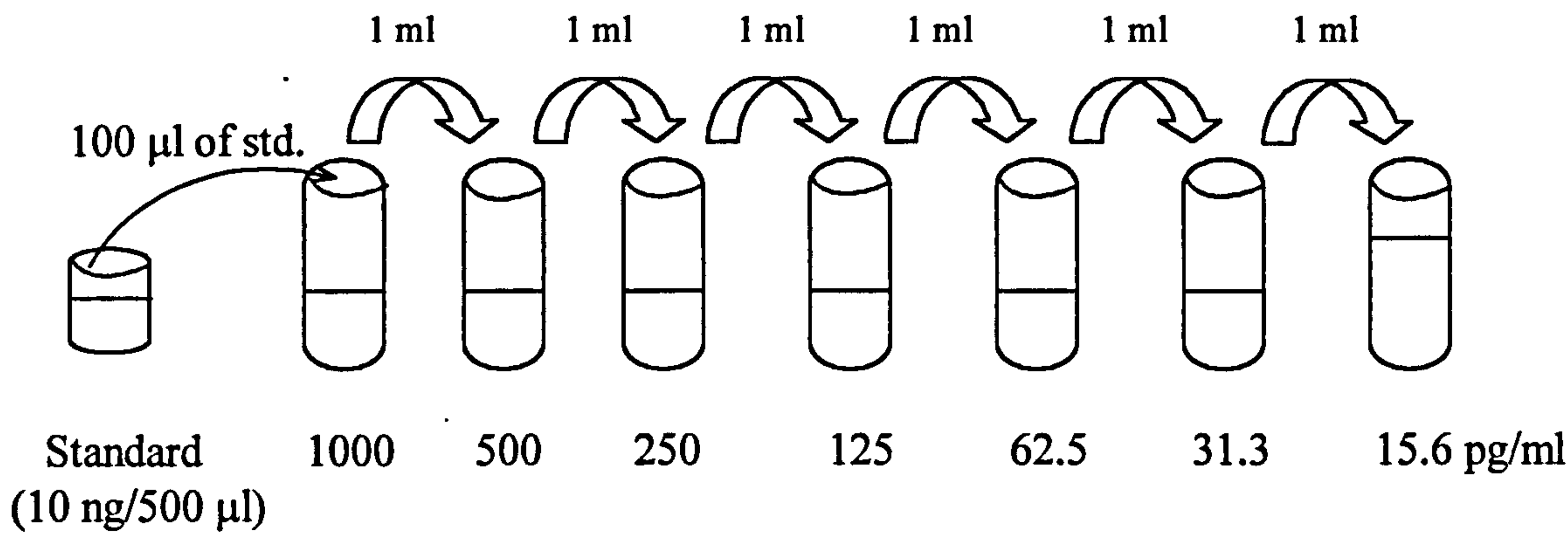
Myometrial tissue lysates from each subject in the different groups as described in section 2.11 were prepared (see section 2.2). Culture medium to be assayed (from section 2.4) was centrifuged at 1000 *g* for 10 min at 4 °C to remove particles. Aliquots of clear supernatant were kept at -70 ° C. In this thesis, myometrial cells were grown in culture medium containing serum, which may contain high levels of TGFβ1. Therefore, the culture medium from control cells was collected to determine the basal levels of TGFβ1.

2.10.3 Activation procedures

TGFβ1 is secreted from cells as a latent complex. To bind to the cell surface receptors *in vivo* it is activated. *In vitro*, the active form of TGFβ1 was prepared by acid treatment followed by neutralisation as described below. All reagents used in the preparation of standards and in the TGFβ1 assay are shown in Appendix 4. 200 µl of hydrochloric acid (1 M) was added to 1 ml of myometrial lysates and culture medium from both control and treated-cells. The lysates and medium were mixed well and incubated for 10 min at room temperature. The pH values of the lysates and medium were checked and adjusted to a pH of 1-2. The acidified samples were neutralised with 200 µl of 1.2 M NaOH/0.5 M HEPES, mixed and were adjusted to pH 7-8. The activated culture medium samples were further diluted (1:2) with assay buffer (see Appendix 4). Because of activation procedures, the dilution of myometrial lysates was 1.4 and that of culture media was 2.8.

2.10.4 Preparation of standards

Clean polystyrene tubes were labelled 1000, 500, 250, 125, 62.5, 31.3 and 15.6 pg/ml. 2 ml of assay buffer was added into 1000 pg tube and 1 ml into each of the other tubes. 100 µl of TGFβ1 standard (10 ng) diluted in 500 µl of reconstitution reagent was added into the 1000 pg tube. Thus, the concentration of TGFβ1 in this tube was 1000 pg/ml. Serial dilutions of this were made as shown below.



2.10.5 Assay procedures

The samples were assayed in duplicate and the procedures are shown in Table 2. All reagents used are shown in Appendix 4.

Table 2. ELISA protocol for TGFβ1

	Blank tube (Zero Std)	Standard tubes	Sample tubes
1. Assay buffer	100 µl	-	-
Standard	-	100 µl	-
Samples			100 µl
2. Cover plate, incubate at 37 ° C for 1 h.			
3. Wash all wells 4 times with wash buffer and blot the plate by tapping briskly on soft paper.			
4. Diluted-detection antibody	100 µl	100 µl	100 µl
5. Incubate at 37 ° C for 1 h and repeat step 3.			
6. Diluted biotinylated second antibody	100 µl	100 µl	100 µl
7. Incubate at 37 ° C for 1 h and repeat step 3.			
8. Diluted streptavidin-HRP conjugated	100 µl	100 µl	100 µl
9. Incubate at 37 ° C for 1 h and repeat step 3.			

Con't

10. TMB substrate	100 µl	100 µl	100 µl
11. Incubate at room temperature for 30 min.			
12. Stop solution	100 µl	100 µl	100 µl
13. Determine optical density (OD) at 450 nm within 30 min.			

2.10.6 Calculation of results

The mean OD of each sample and standard was calculated. The mean OD of the zero standard was subtracted from each mean value of sample and standard. A linear dose-response standard curve was constructed by plotting a log/log plot of mean OD (y axis) against standard TGFβ1 concentration (x axis). The values of samples were read directly from the standard curve or calculated from the linear regression analysis formula obtained from the curve. The concentrations of total TGFβ1 in myometrial tissues are reported as pg/g tissue wet weight. For cultured cells, the total amount of TGFβ1 in the culture media in each well was divided by the total amount of protein from the same well. The total levels of TGFβ1 were then reported as the mean of four batches of cells and expressed as pg/µg cell protein.

2.11 Intracellular cAMP enzyme-immunoassay

Levels of intracellular cAMP in cultured cells were measured using cAMP enzyme immunoassay kits from Amersham.

2.11.1 Sample preparation

After forskolin treatment, the culture medium was aspirated. The cells in each well were lysed in 300 µl of lysis reagent 1B supplied by the manufacturer. The plates were placed on a plate shaker and shaken for 10 min to facilitate cell lysis. Lysed cells were processed immediately for the immunoassay or kept at -20 °C until used.

2.11.2 Preparation of working standards

Polystyrene tubes were labelled 12.5, 50, 100, 200, 400, 800 and 1600 fmol. 500 µl of lysis reagent 1B was pipetted into all tubes. Stock standard (32 pmol/ml) at a volume of 500 µl was pipetted into the 1600 fmol tube. The standard solution was mixed thoroughly and 500 µl of standard from the 1600 fmol tube was transferred to the 800 fmol tube. The serial dilution steps were repeated with the remaining tubes and a 100 µl aliquot from each dilution provided eight standards from 12.5-1600 fmol. 100 µl of stock standard was used as the top standard concentration of cAMP (3200 fmol).

2.11.3 Assay procedures

The assay was performed in duplicate and the protocol is shown in Table 3. Reagents and reagent preparations are shown in Appendix 5.

Table 3. Enzyme immunoassay protocol for cAMP

	Blank Tube (B)	Non-specific binding tube (NSB)	Zero standard tube (B ₀)	Standard tubes	Sample tubes
Lysis reagent 1B	-	100 µl	100 µl	-	-
Lysis reagent 2B	-	100 µl	-	-	-
Standards	-	-	-	100 µl	-
Samples	-	-	-	-	100 µl
Antiserum			100 µl	100 µl	100 µl
Cover plate and incubate at 4 °C for 2 h					
Peroxidase- conjugate	-	50 µl	50 µl	50 µl	50 µl
Cover plate and incubate at 4 °C for 1 h, aspirate and carefully wash all wells four times with 400 µl of wash buffer					
TMB substrate	150 µl	150 µl	150 µl	150 µl	150 µl

Con't

Cover plate and incubate on a shaker for exactly 1 h at room temperature					
1 M sulfuric acid	100 µl	100 µl	100 µl	100 µl	100 µl
Determine the optical density at 450 nm within 30 min					

2.11.4 Calculation of results

The mean OD of each sample and standard was calculated. The percentage bound for each standard and sample was calculated using the following formula:

$$\% B/B_0 = \frac{(\text{standard or sample OD-NSB OD}) \times 100}{(\text{zero standard OD-NSB OD})}$$

A standard curve was generated by plotting % B/B₀ as a function of the log cAMP concentration. Thus, % B/B₀ was plotted (y-axis) against fmol cAMP/well (x-axis) on semi-log graph. The concentrations of samples were read directly from the standard curve. The mean of intracellular cAMP concentration (triplicate experiments) from each batch of cells was expressed as fmol/µg cell protein.

2.12 Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Comparisons of means between two groups were analysed with Student's *t* test. Multiple comparisons were performed using analysis of variance (ANOVA) with a Bonferroni correction. Where appropriate, Kruskal Wallis was used and indicated in the figure legend. *P* values < 0.05 were considered significant. Asterisks (* and #) indicate the levels of significance.

Chapter 3

Gestational changes in the levels of TGF β 1 and TGF β receptor types I and II in the human myometrium

The ability of the myometrium to contract efficiently at term may be determined by a number of specific contraction associated proteins (CAPs) that control the mechanism of contraction (Lye, 1996). By triggering the synthesis of these proteins, the myometrium can be transferred from a state of overall quiescence into one primed for activation (Lopez Bernal *et al.* 1995). As term proceeds, a number of CAPs such as oxytocin receptors (Kimura *et al.* 1996), gap junctions (Chow & Lye, 1994), ion channels (Boyle & Heslip, 1994; Mershon *et al.* 1994) and the ryanodine sensitive intracellular Ca²⁺ release mechanism, RyR2 (Awad *et al.* 1997), are increased within the human myometrium. The nature of the signals that trigger the synthesis of these CAPs *in vivo* is not known. It has been shown that RyR2 is up regulated by TGF β 1 in cultured myometrial smooth muscle cells (Awad *et al.* 1997). This leads to the idea that TGF β 1 could play a similar role in the activation of RyR2 gene in the process of myometrial preparation *in vivo*.

TGF β is a multifunctional cytokine, which exerts its actions via receptor Types I, Type II and Type III (Massague, 1992; Massague & WeisGarcia, 1996). The intracellular processes linking TGF β and TGF β receptor activation can be complex. One action of TGF β is the activation or inhibition of gene expression (Heldin *et al.* 1997). If TGF β 1 plays a similar role in the activation of specific CAP genes in the pregnant myometrium, then it is important to demonstrate the presence of TGF β 1 and TGF β receptors in the myometrium. The present experiments were performed to explore the idea that the TGF β system might form part of a signalling cascade *in vivo*.

3.1 TGF β receptor expression

Immunohistochemistry was used to determine whether TGF β receptors were expressed in uterine smooth muscle cells in myometrial samples collected from non-pregnant, term pregnant non-labouring and term spontaneous labouring women. Figure 3.1 (A, B and C) show photomicrographs of tissue sections of human myometrium from non-pregnant, pregnant-non labouring and spontaneous labouring women respectively. Each section illustrates (a) control sections (b) sections stained for type I TGF β receptor (T β RI) (c) staining for type II TGF β receptor (T β RII) and (d) staining for type III TGF β receptor (T β RIII). In each of the three patient groups, TGF β receptor types I and II were detected in the myometrial smooth muscle cells. No expression of type III TGF β receptor was detected in any of the tissues studied.

Western immunoblotting was subsequently employed to quantitate the relative amounts of TGF β receptors expressed in tissue lysates prepared from the different myometrial tissues. Figure 3.2A shows nitro-cellulose blots stained with antibodies to TGF β receptor types I and II in non-pregnant, term pregnant non-labouring and spontaneous labouring tissues respectively. Blots were also incubated with an antibody to the G protein sub-unit, G β , as a loading control as the expression of this G-protein sub-unit remains constant in the human myometrium during pregnancy and labour (Europe Finner *et al.* 1994). As in Figure 3.1, the protein for TGF β receptor types I and II were detected in all samples but no protein corresponding to TGF β receptor type III was found. The relative expression of receptors in each tissue was analysed using a scanning densitometer. The data for receptor types I and II expression in the myometrial tissues after normalising for loading (using the G β) are shown in Figure 3.2B. The levels of TGF β receptor types I and II expression in non-pregnant samples were arbitrarily assigned as 100 %. The amounts of receptor in term pregnant non-labouring tissues and spontaneous labouring myometrium are expressed relative to this value. The expression levels of TGF β receptor types I and II were significantly increased by

168 \pm 19 % (n = 6) and 162 \pm 22 % (n = 7) in term pregnant non-labouring tissues ($P < 0.05$) compared to those in non-pregnant myometrium. The expression of both receptor types obtained from term spontaneous labouring myometrium were 93 \pm 12 % (n = 6) and 85 \pm 11 % (n = 7), respectively. These values were significantly lower than those in pregnant non-labouring tissues ($P < 0.01$) which suggest that there is less protein for both receptor types I and II in spontaneous labouring myometrium.

In a further series of experiments, the relative levels of T β RI expression were also compared in non-contraction myometrial samples obtained at 31-35 weeks and those obtained from term pregnant-non labouring samples. Figure 3.3A demonstrates a Western blot of T β RI in myometrial tissue lysates from three pre-term (lane 1-3) and three term non-labouring myometrial samples (lane 4-6). Figure 3.3B shows the mean data of T β RI expression analysed from five pre-term and seven term myometrial tissue samples. The expression of T β RI obtained from pre-term myometrium was 53 \pm 7 %. The results show that T β RI expression in myometrium is significantly lower in pre-term compared to term pregnancy (100 %, $P < 0.01$).

Figure 3.1 Localisation of TGF β receptors in uterine smooth muscle cells in the human myometrium.

Tissue sections from non-pregnant, pregnant non-labouring and spontaneous labouring myometrium are illustrated (A, B and C respectively, which is representative of two experiments). For each tissue a control, without TGF β receptor antibody and counterstained only with haematoxylin is shown (a). The presence of TGF β receptor types I, II and III are illustrated in sections (b), (c) and (d) respectively. Positive staining is represented by the distinctive brown colour within the myometrial smooth muscle cells. This experiment was repeated twice in each sample group ($n = 2$).

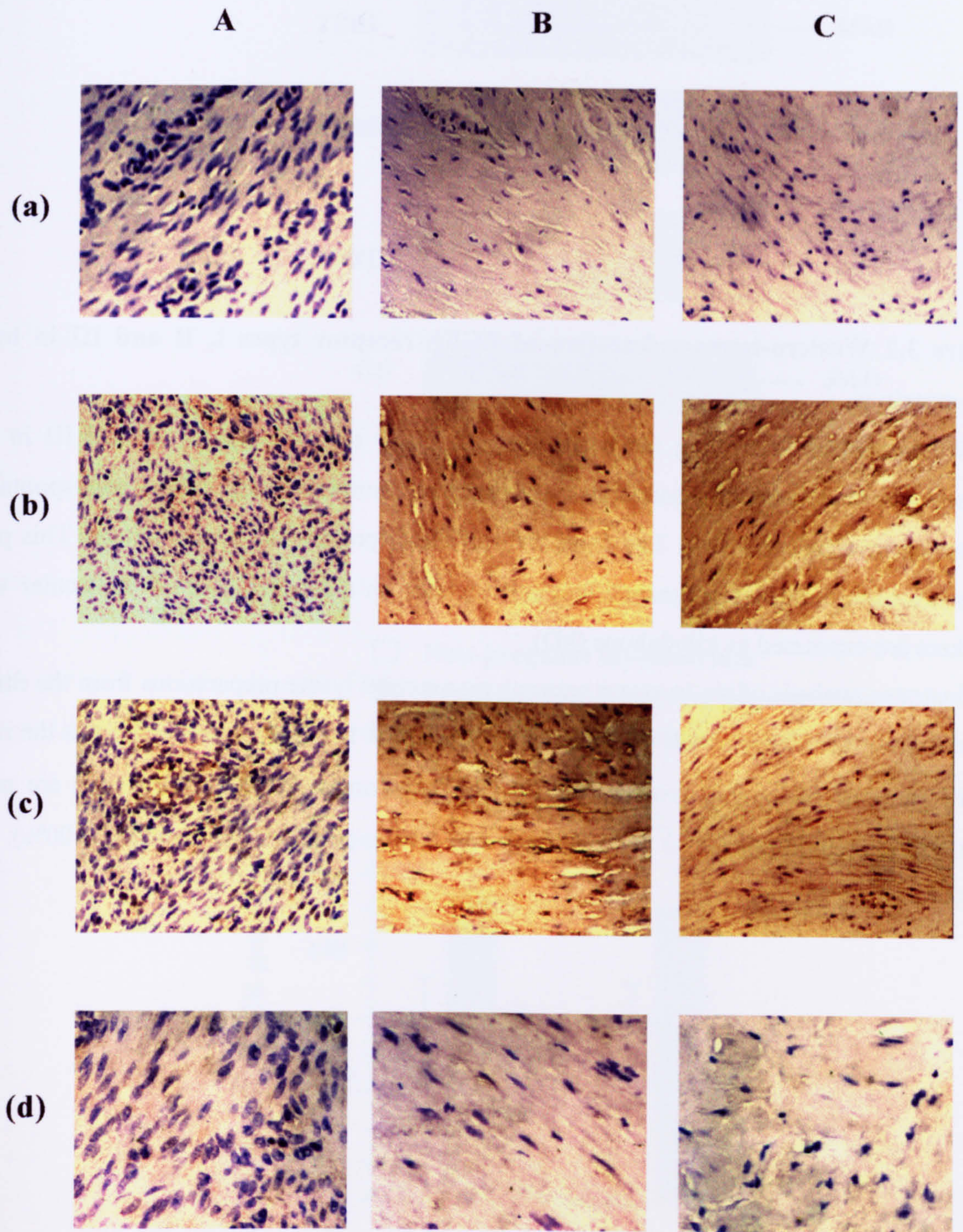
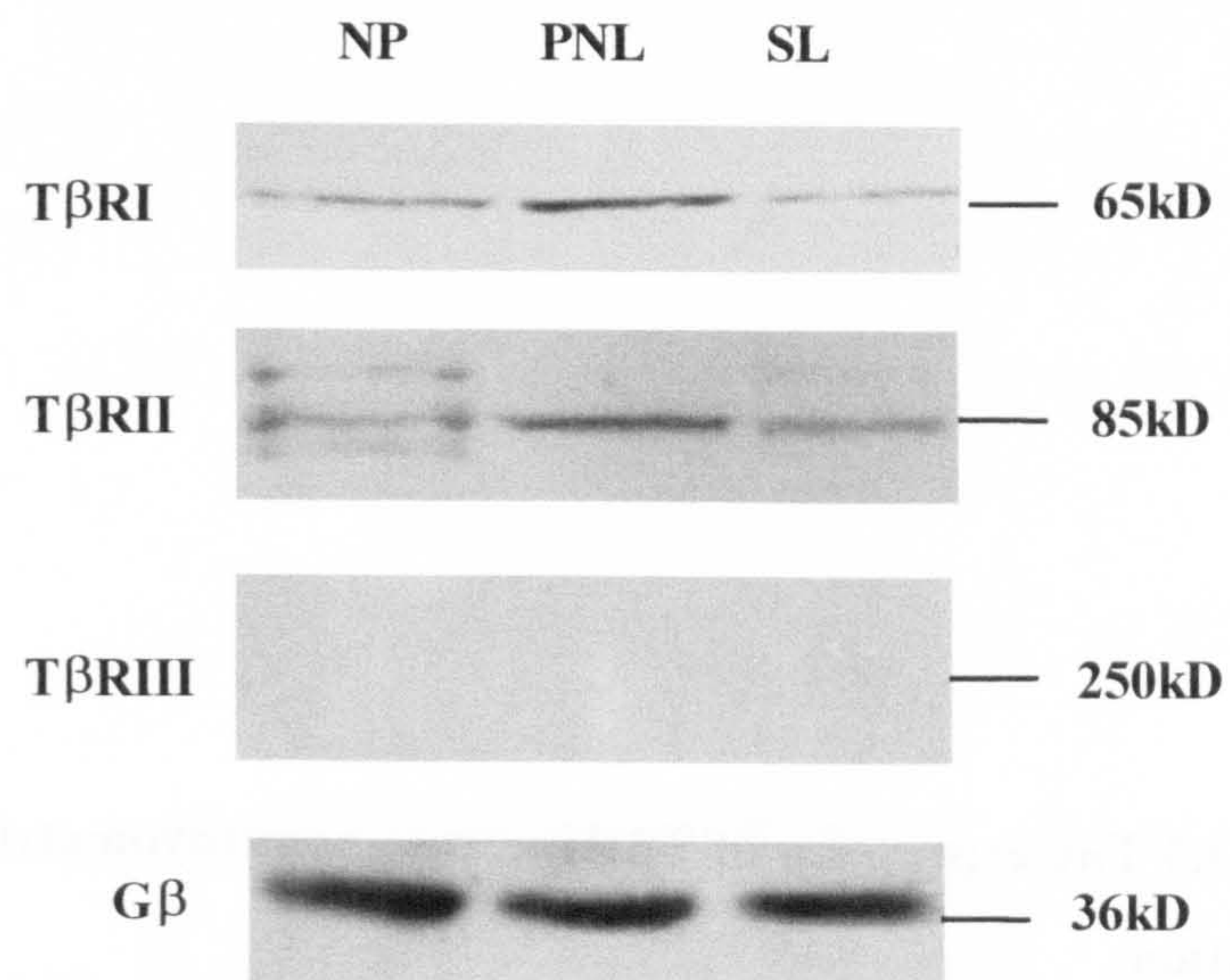


Figure 3.2 Western-immunodetection of TGF β receptor types I, II and III in human myometrium

A. Western blots illustrating the expression of TGF β receptor types I, II and III in tissue lysates prepared from non-pregnant (NP), pregnant non-labouring (PNL) and spontaneous labouring myometrium (SL). The expression of the G-protein G β is also shown. This protein does not change during pregnancy and can be used as a loading control. Molecular weight markers are expressed as kilodaltons (kD).

B illustrates analysis of six to seven separate myometrial lysate preparations from the different patient groups. Relative levels of TGF β receptor in each tissue were determined as the ratio of TGF β receptor protein/G β protein measured by densitometry. The values shown are mean \pm SEM. and expressed relative to the receptor levels of non-pregnant myometrium (100%). *, $P < 0.05$. **, $P < 0.01$.

A.



B.

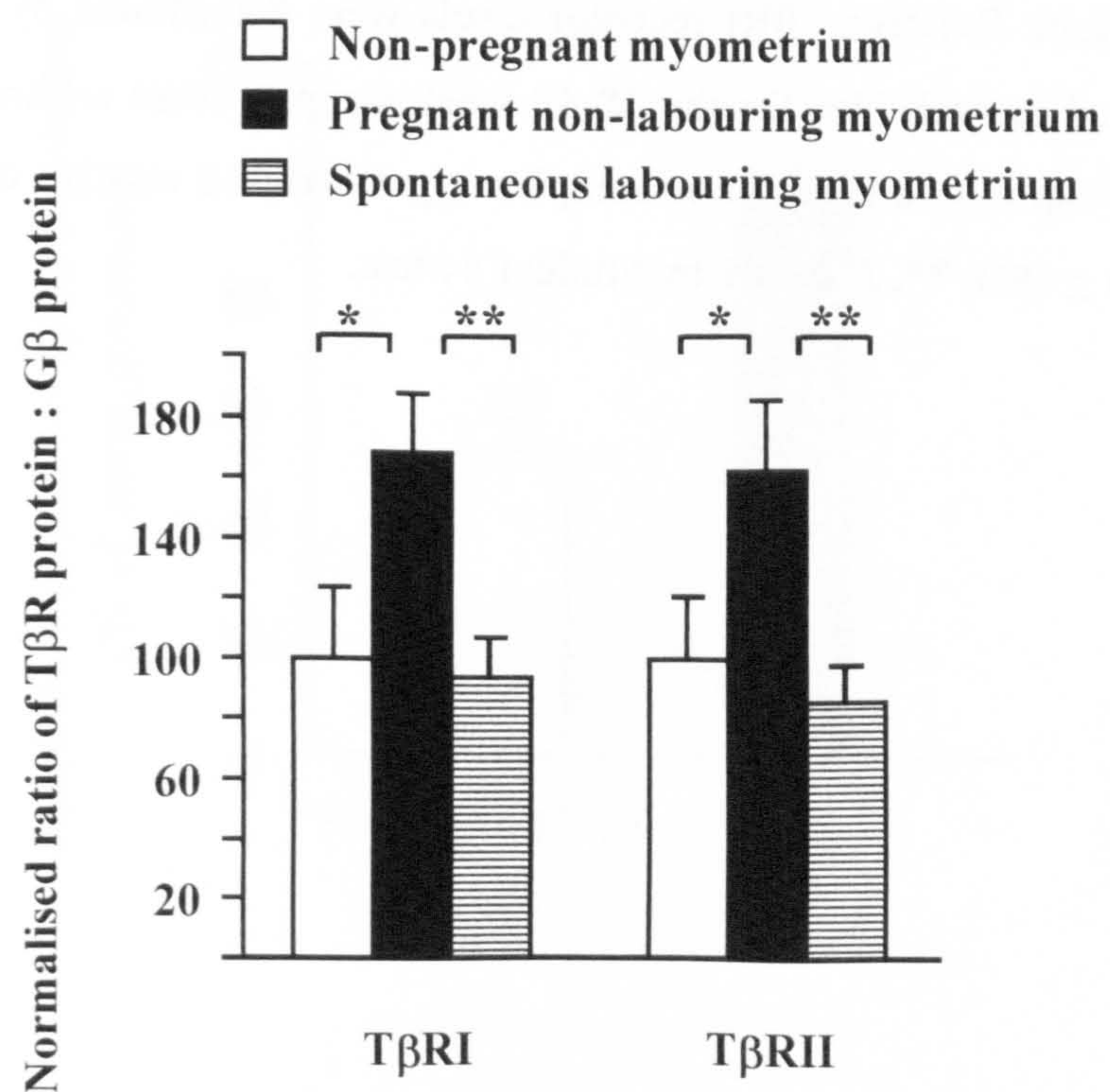
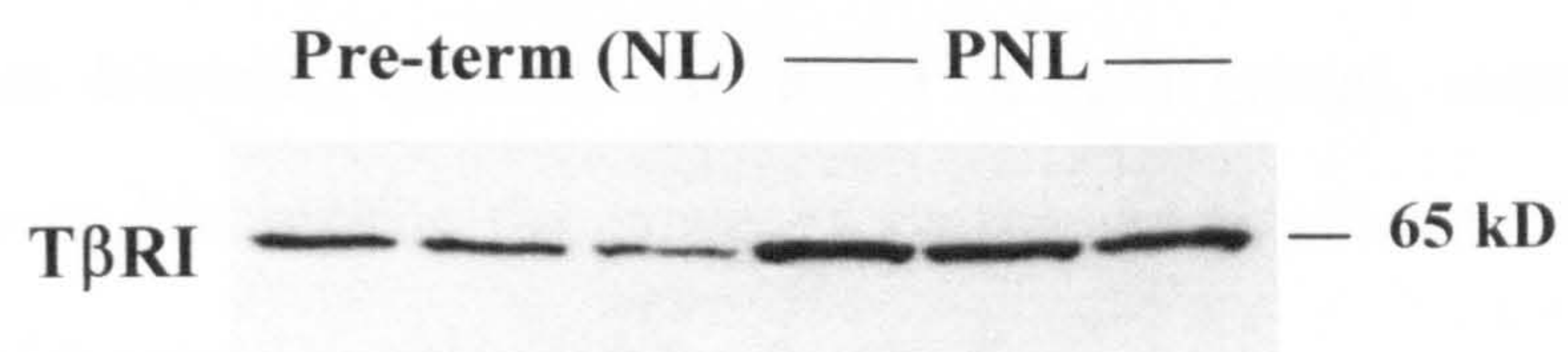


Figure 3.3 The expression of T β RI in pregnant myometrium at 31-35 and 38-40 weeks of gestation

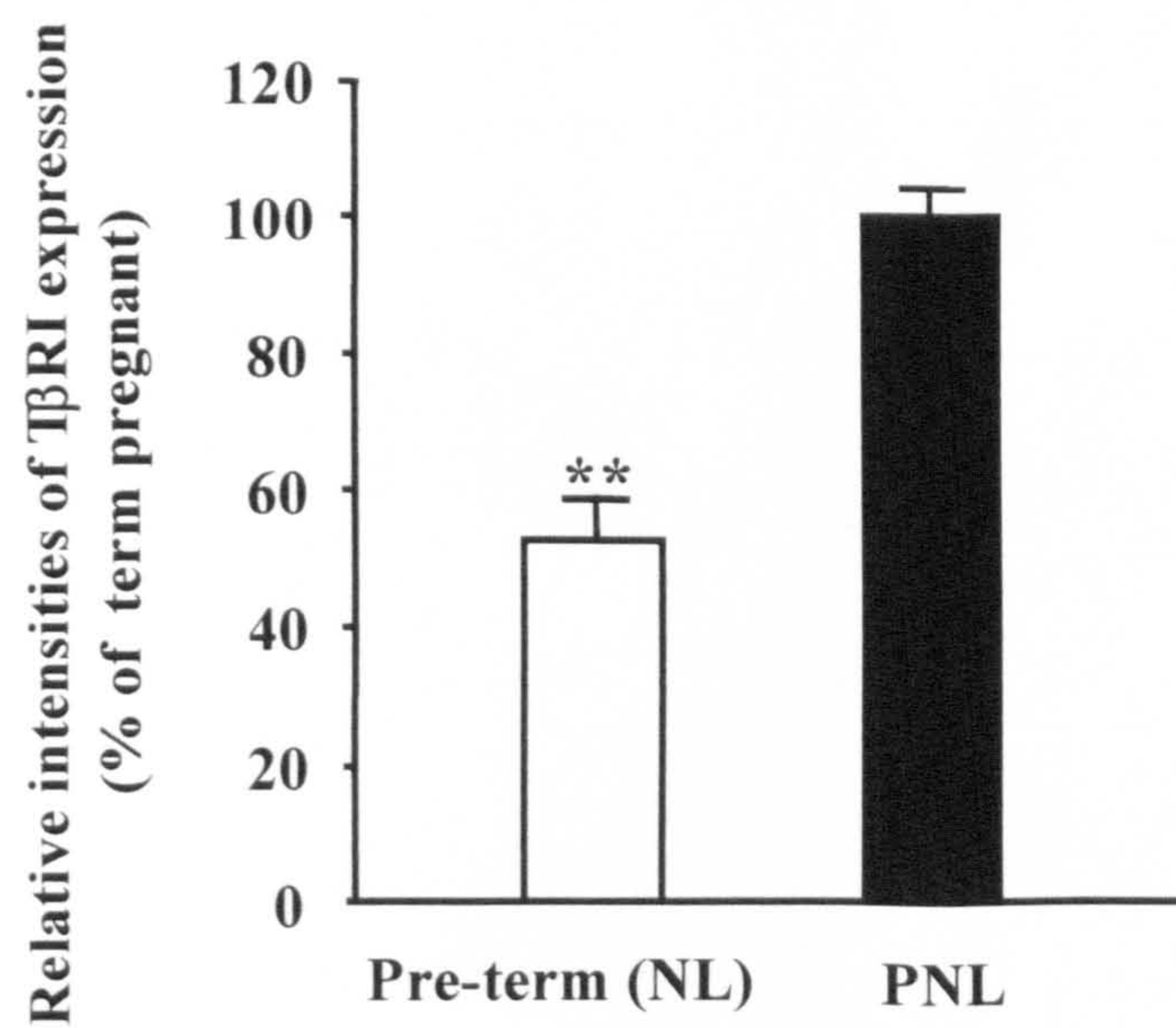
A Western blots illustrating the expression of T β RI in lysates prepared from myometrial tissues from pre-term non-labouring (31-35 weeks) and pregnant term non-labouring (PNL, 38-40 weeks) myometrium.

B illustrates analysis of five samples from pre-term and seven samples from term pregnant myometrium. Relative T β RI receptor levels were determined by densitometry. The levels determined in each blot for the 38-40 week samples were arbitrarily set at 100% and the levels of expression in pre-term samples expressed as a relative change. The values shown are mean \pm SEM. **, $P < 0.01$ by Student's t test.

A.



B.

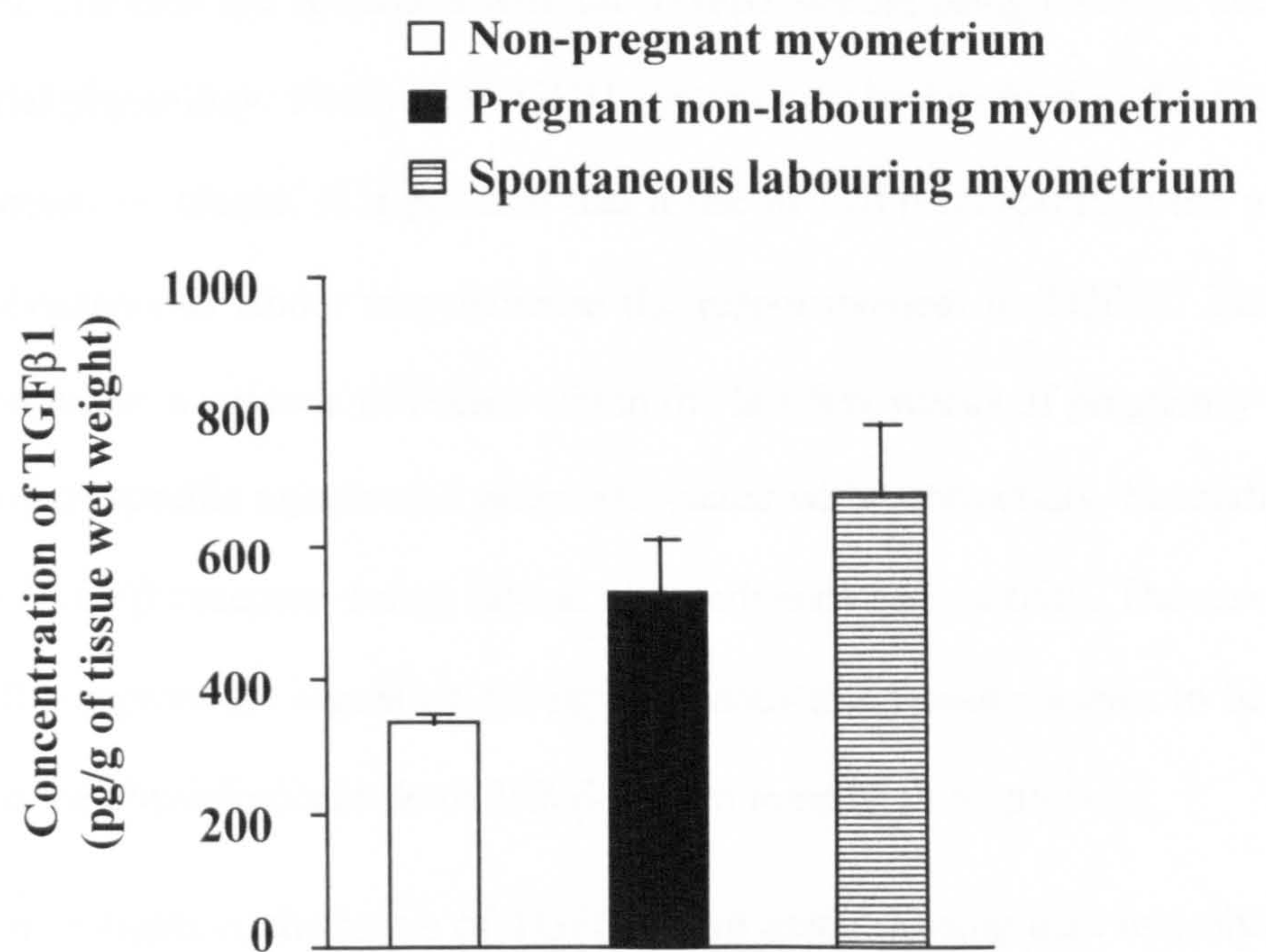


3.2 *TGFβ1* expression in the myometrium

Because TGFβ receptors were detected in the myometrium at the end of pregnancy and in spontaneous labouring myometrium, it was then important to establish whether TGFβ could be measured in these tissues. The presence of both cytokine and receptor are essential if this system is to have a physiological role. Figure 3.4 illustrates measurements of the tissue concentrations of the total TGFβ1 (latent plus active) in non-pregnant, term pregnant non-labouring and term spontaneous labouring myometrium using ELISA. The amount of TGFβ1 was increased from 334 ± 10 pg/g of tissue wet weight in non-pregnant myometrium to 534 ± 73 and 674 ± 106 pg/g of tissue wet weight in pregnant non-labouring and spontaneous labouring myometrium ($n = 3$ in each group), respectively. However, these values were just outside the range of statistically significant difference between groups because of the small sample size ($P = 0.06$).

Figure 3.4 TGFβ1 in human myometrium

Measurements of the total concentrations of TGFβ1 in non-pregnant, pregnant non-labouring and spontaneous labouring myometrium. Concentrations are expressed as pg/g of tissue wet weight. Values are mean \pm SEM (n = 3 for each patient group). Data were analysed with Kruskal Wallis.



3.3 Discussion

The major observations in this study are 1) that the levels of TGF β receptor types I and II changed with pregnancy and 2) that the levels of TGF β 1 in the myometrium are not significantly altered. These observations are analogous to the change reported in the levels of oxytocin receptor prior to labour without any change of oxytocin levels. Thus, it may be a general principle that the myometrium regulates its functions at the level of surface membrane receptors rather than by endocrine or paracrine hormone levels. These observations support the idea that TGF β 1 may operate as an autocrine signal in the myometrium. The data are consistent with the TGF β 1 system being involved in two aspects of myometrial physiology. Firstly the TGF β 1 system may be involved in the preparation of the myometrium for labour. It is possible that a rise in TGF β receptors in the myometrium preceding spontaneous labour may increase the responsiveness to TGF β 1. These changes may function within a cascade of events within the last few weeks of pregnancy to facilitate the activation of specific myometrial genes associated with contractility. Secondly the down regulation of TGF β receptor during labour may influence contractility. The mechanisms by which TGF β receptors are regulated during pregnancy and labour remains to be elucidated. Furthermore the physiological role of TGF β system remains to be proven.

In this investigation, the levels of TGF β 1 were assayed using acid hydrolysis and only total levels of TGF β 1 (latent + active) were determined. Generally, it is only active TGF β 1 that can bind to the receptor. Although we could not detect any changes in total TGF β 1 levels, it is possible that there could be a change in the amount of active TGF β 1 produced in the myometrium. The total levels of TGF β 1 may be increased but only three samples were studied. With such small numbers we could not exclude anything but a large change. With this perspective further experiments would be desirable to address this specific question. Alternative approaches for the determination of the amount of TGF β 1 expression such as Western analysis for the protein or RT-PCR for RNA determination was considered.

Bioassay techniques were also considered to determine the amount of active TGF β 1 in the future experiments.

A number of other CAPs such as oxytocin and endothelin receptors are found to be increased in the myometrium at term (Kimura *et al.* 1996; Wolff *et al.* 1996). Conversely, the number of angiotensin receptors is decreased as term approaches (Matsumoto *et al.* 1996). Thus it would appear that a common mechanism that could influence myometrial function would be to regulate 'up' or 'down' the expression of essential receptors. In this way, the myometrium can alter its function from a weakly contractile and poorly excitable tissue to one capable of powerful co-ordinated contractions.

TGF β has been postulated to be a potential regulator of many functions in the non-pregnant uterus and in early pregnancy. It has been reported that levels of expression of TGF β receptors and TGF β change during the menstrual cycle (Chegini *et al.* 1994). The role of oestrogen and progesterone has been suggested to account for these variations in the human uterus (Chegini *et al.* 1994). Human myometrial cells *in vitro* have been shown to synthesise and release TGF β (Chegini *et al.* 1996; Tang *et al.* 1997). In turn, it has been shown that the amount of TGF β synthesised can be increased by gonadotrophin releasing hormone (GnRH), 17 β oestradiol and medroxyprogesterone acetate (Chegini *et al.* 1996).

TGF β has also been reported in the murine uterus at implantation, where it may be involved in synchronising embryonic development (Tamada *et al.* 1990; Das *et al.* 1992). In the non-pregnant ovariectomised mouse uterus, the expression of TGF β can be transiently increased by the injection of oestrogen (Das *et al.* 1992). It has been reported in other cell types in animals that the expression of TGF β receptors can be influenced by several hormones (Cochet *et al.* 1988; Kim *et al.* 1996). For example, ACTH up-regulates TGF β receptors in bovine adrenocortical cells (Cochet *et al.* 1988) and androgens affect receptor expression in the ventral prostate in rat (Kim *et al.* 1996).

Taken together these observations indicate that the TGF β signalling system is under hormonal regulation. This may also be the case in late human pregnancy. TGF β receptors are however unlikely to have a role in the direct activation of contractions. It is more likely that they are associated with activation of myometrial cell growth and differentiation in preparation for parturition. Thus, it can be speculated that the myometrium is prepared for term as a result of a cascade of events involving the sequential expression of signals, receptors and contraction associated proteins.

The activation of the TGF β signalling pathway involves binding of TGF β to the type II receptor, resulting in the formation of a complex between type I and type II receptors. The consequence of this binding is the phosphorylation of the type I receptor (Heldin *et al.* 1997; Massague & WeisGarcia, 1996). However, in some instances TGF β can activate cells that lack detectable level of type II receptor (Chen *et al.* 1993) via activation of type I receptors alone (Wieser *et al.* 1995). It has been shown that a MAD-related family of signal transducers is required for intracellular events in the TGF β pathway (for reviews see (Wrana, 1998; Derynck & Feng, 1997)). This includes the *Drosophila Mothers against dpp gene (Mad)* and its homologue, *Sma* in *C. elegans*. In human, a group of vertebrate homologues of *Sma* and *Mads*, designated *Smads*, in the cytoplasm is known to be a potential signalling mediators of TGF β (Wrana & Pawson, 1997; Heldin *et al.* 1997). Other proteins using the type I receptor such as FK506-binding protein may also modulate receptor signalling (Heldin *et al.* 1997; Massague & WeisGarcia, 1996). However, the mechanisms underlying the TGF β signals in the human myometrium are still unknown.

Chapter 4

Complex interactions between sex steroids and cytokines in the human pregnant myometrium: evidence for an autocrine signalling system at term pregnancy

Cytokines have been thought to play a role not only in the mechanisms of pre-term delivery but also normal labour (Romero *et al.* 1990; Steinborn *et al.* 1996). Recently, there have been several reports showing that the concentrations of tumour necrosis factor- α (TNF α), IL-1 and IL-8 in the human myometrium are increased towards term (Osmers *et al.* 1995; Kemp *et al.* 1998). Thus, it can be hypothesised that a cytokine cascade is involved in the preparation of human myometrium for term.

It has been shown that there are two types of IL-1 receptor (Sims *et al.* 1989; McMahan *et al.* 1991). These are termed IL-1 receptor types I (IL-1RI) and type II (IL-1RII). IL-1RI has been found in many cell types including endothelium, fibroblast, decidua and smooth muscle cells (Sims *et al.* 1989; Sims & Dower, 1994; Ammala *et al.* 1997; Whittle *et al.* 1999) and has been considered a signal transducing receptor (Colotta *et al.* 1993; Sims *et al.* 1993). However, there is no data to assess the possibility that the myometrium expresses receptors for IL-1. Two types of receptors for IL-8, types A (IL-8RA) and B (IL-8RB), have been demonstrated in myometrium before the initiation of labour but there has been no estimate of the levels of IL-8 receptor expression (el Maradny *et al.* 1996b). In most cells IL-8 can bind to both receptors and each receptor has different functions (Chuntharapai & Kim, 1995). In neutrophils, for example, IL-8RA plays an active role in the initiation of neutrophil migration at the site of inflammation. In contrast, IL-8RB mediates IL-8 signals outside the inflammatory site (Chuntharapai & Kim, 1995).

Because cytokines work through their specific receptors, the first study in a series of experiments was to demonstrate whether the human myometrium expresses IL-1RI and IL-8RB receptors and to identify if levels change with gestation.

4.1 IL-1 and IL-8 receptor expression

In each series of experiments, the level of expression in the non-pregnant myometrium was arbitrarily set to 100 % and changes in term pregnant non-labouring and spontaneous labouring tissues expressed relative to these values. Figure 4.1A shows typical Western blots using an antibody raised against the IL-1 receptor on samples of non-pregnant, pregnant non-labouring and spontaneous labouring tissues. Figure 4.1B shows accumulated data from a densitometric analysis, obtained from eight tissue samples in each group. The highest level of IL-1RI expression was found to be in the non-pregnant samples. Compared to the non-pregnant tissue, the receptor expression was significantly reduced to $71 \pm 8 \%$ ($P < 0.05$) and $40 \pm 7 \%$ ($P < 0.001$) in the term pregnant non-labouring and spontaneous labouring samples, respectively. There was also a significant difference in the expression levels between the pregnant and spontaneous labouring tissue ($P < 0.01$).

Figure 4.2A shows an example of a Western blot examining the expression of IL-8RB in the same group of myometrial samples. As can be seen in Figure 4.2B, in contrast to the IL-1 receptor, the expression of IL-8 RB was highest in the term pregnant non-labouring samples ($196 \pm 24 \%$ of the non-pregnant level, $P < 0.01$). In the spontaneous labouring myometrium, the expression of IL-8RB was found to be reduced to $59 \pm 10 \%$ compared to the non-pregnant control but this is not statistically different. However, this level was significantly lower than the level obtained from the pregnant non-labouring myometrium ($P < 0.001$).

As shown previously in chapter 3, TGF β receptors change during late pregnancy, raising the question “what are the signals in the cytokine cascade that influence the expression of TGF β receptors?”. It has been reported in many cell types that cytokines can

affect the expression of other cytokines and cytokine receptors (Balkwill & Burke, 1989; Arai *et al.* 1990; Foxwell *et al.* 1992). Cytokines can also regulate the expression of their own receptors. It is conceivable that complex cytokine interactions may take place in the human myometrium. It is not possible to investigate interactions between cytokines and the expression of other cytokines and receptors during pregnancy *in vivo*. Therefore, a series of experiments were carried out in cultured human myometrial smooth muscle cells isolated from non-pregnant myometrium to determine the possibility that IL-1 and IL-8 might influence the expression of TGF β 1 and T β RI. The possible interactions between these cytokines were also investigated.

Figure 4.1 Western immunodetection of IL-1 receptor type 1 (IL1-RI) expression in the human myometrium

A. Western blots illustrating the expression of IL1-RI in tissue lysates prepared from non-pregnant (NP), pregnant non-labouring (PNL) and spontaneous labouring myometrium (SL).

B illustrates analysis of 8 NP, 8 P and 8 SL tissue samples. Relative IL-1 receptor levels in each tissue was determined by densitometry. The levels determined in each blot for the NP samples were arbitrarily set at 100% and the levels of expression in the P and SL samples expressed as a change relative to the NP level. The values shown are mean \pm SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 4.2 Western immunodetection of IL-8 receptor type B (IL8-RB) expression in the human myometrium

A. Western blots illustrating the expression of IL8-RB in tissue lysates prepared from non-pregnant (NP), pregnant non-labouring (P) and spontaneous labouring myometrium (SL). B illustrates analysis of 7 NP, 7 P and 7 SL tissue samples prepared from different patient groups. Relative IL-8 receptor levels in each tissue were determined by densitometry. The levels determined in each blot for the NP samples were arbitrarily set at 100% and the levels of expression in the P and SL samples expressed as a change relative to the NP level. The values shown are mean \pm SEM. **, $P < 0.01$; ***, $P < 0.001$.

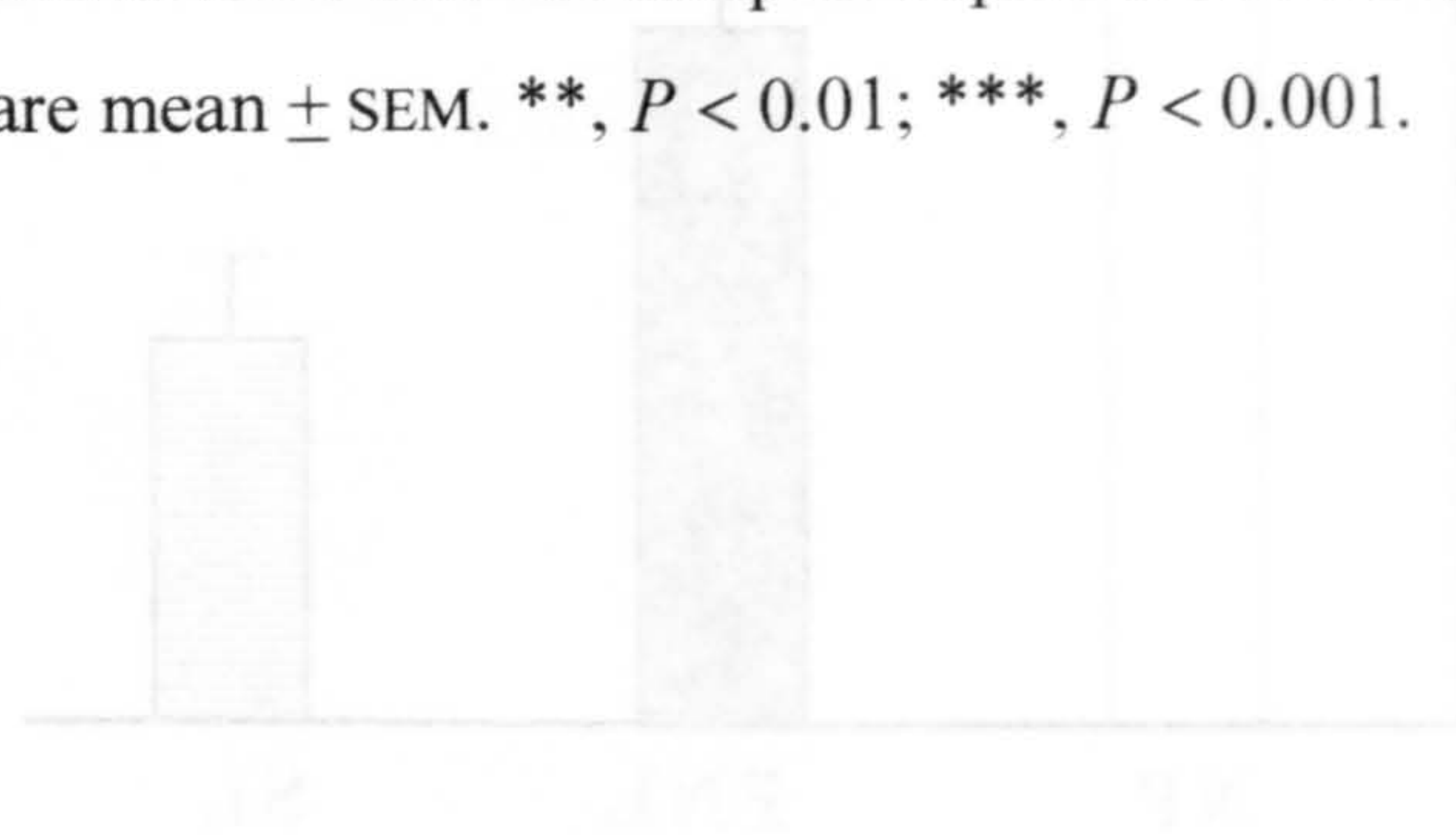


Figure 4.1 Western immunodetection of IL-1 receptor type 1 (IL1-R1) expression in the human myometrium

A. Western blots illustrating the expression of IL1-R1 in tissue lysates prepared from non-pregnant (NP), pregnant non-labouring (PNL) and spontaneous labouring myometrium (SL).

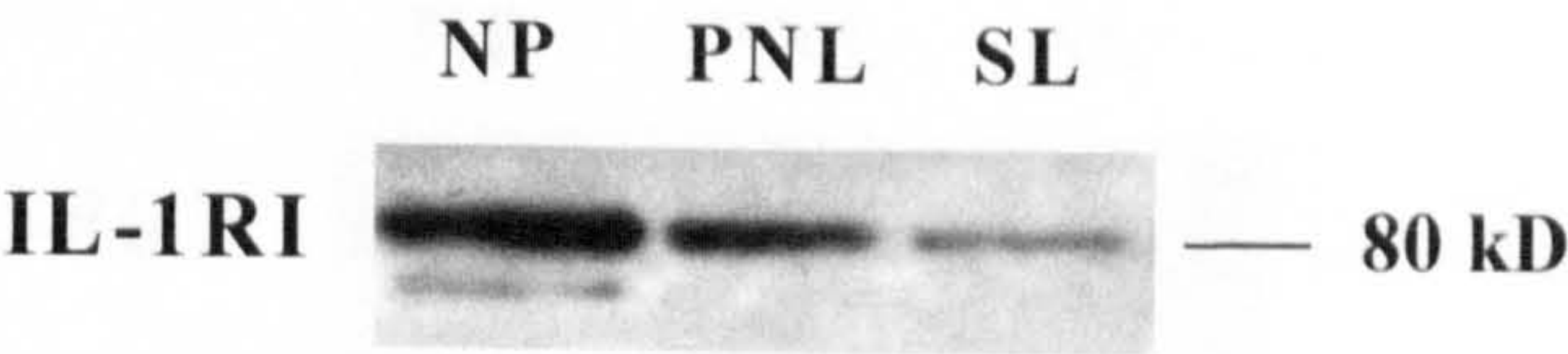
B illustrates analysis of 8 NP, 8 P and 8 SL tissue samples. Relative IL-1 receptor levels in each tissue was determined by densitometry. The levels determined in each blot for the NP samples were arbitrarily set at 100% and the levels of expression in the P and SL samples expressed as a change relative to the NP level. The values shown are mean \pm SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 4.1 Western immunodetection of IL-1 receptor type 1 (IL1-RI) expression in the human myometrium

A. Western blots illustrating the expression of IL1-RI in tissue lysates prepared from non-pregnant (NP), pregnant non-labouring (PNL) and spontaneous labouring myometrium (SL).

B illustrates analysis of 8 NP, 8 P and 8 SL tissue samples. Relative IL-1 receptor levels in each tissue was determined by densitometry. The levels determined in each blot for the NP samples were arbitrarily set at 100% and the levels of expression in the P and SL samples expressed as a change relative to the NP level. The values shown are mean \pm SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

A.



B.

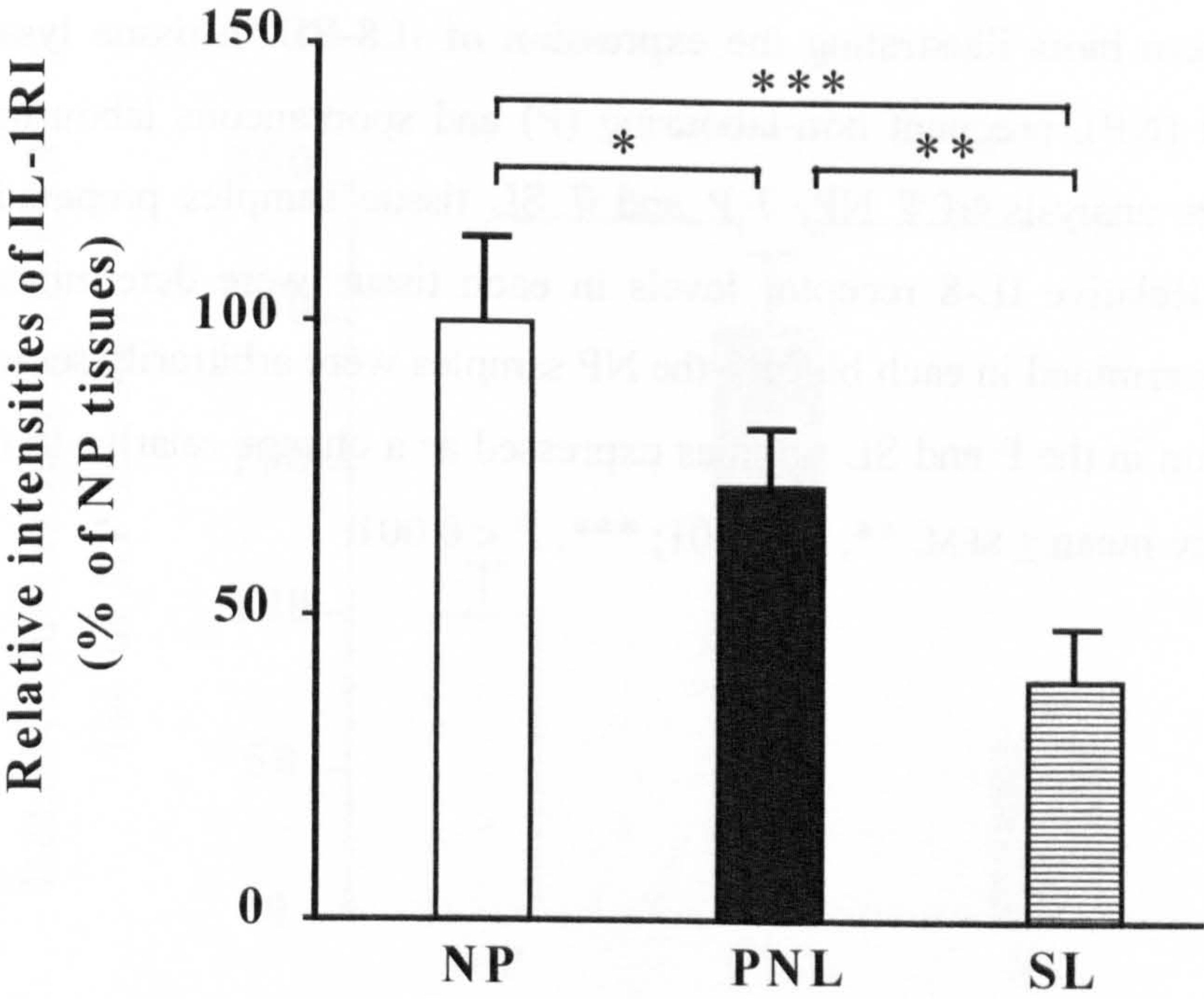
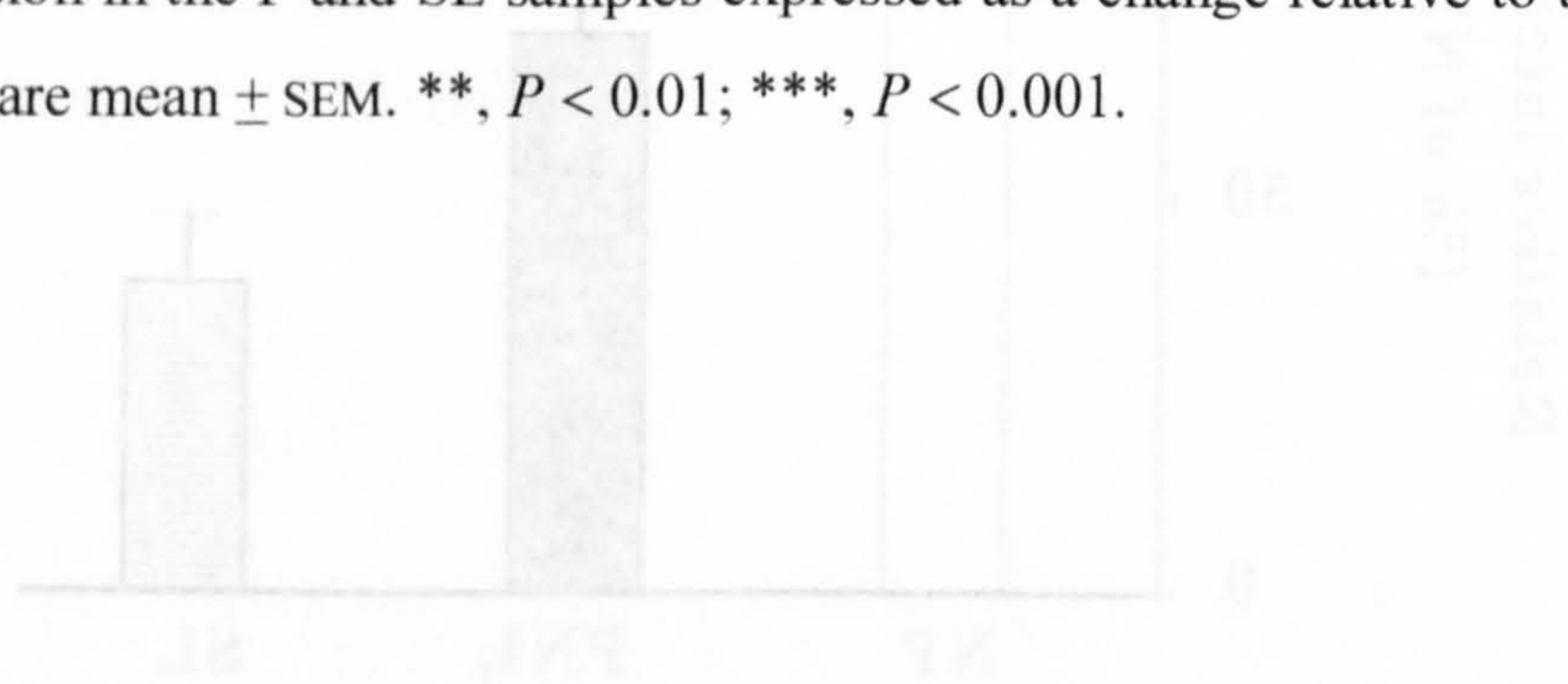
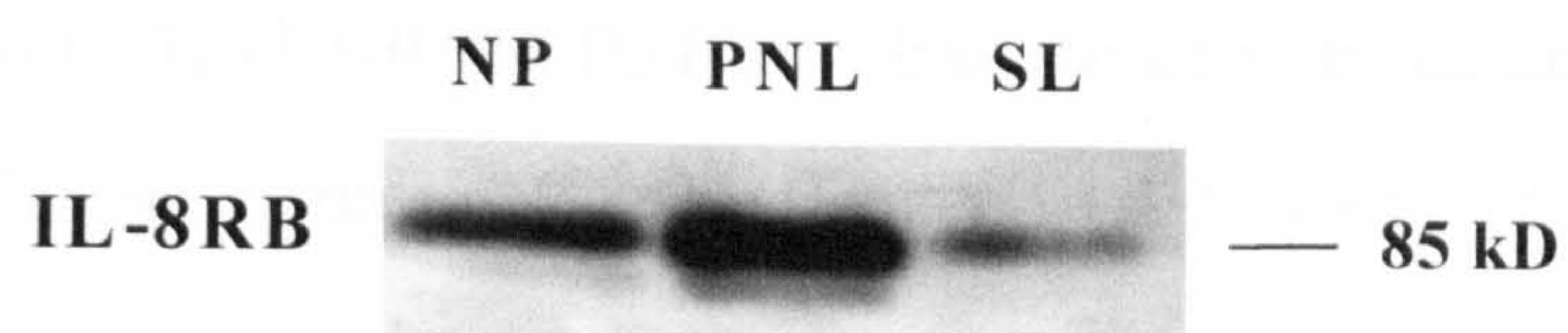


Figure 4.2 Western immunodetection of IL-8 receptor type B (IL8-RB) expression in the human myometrium

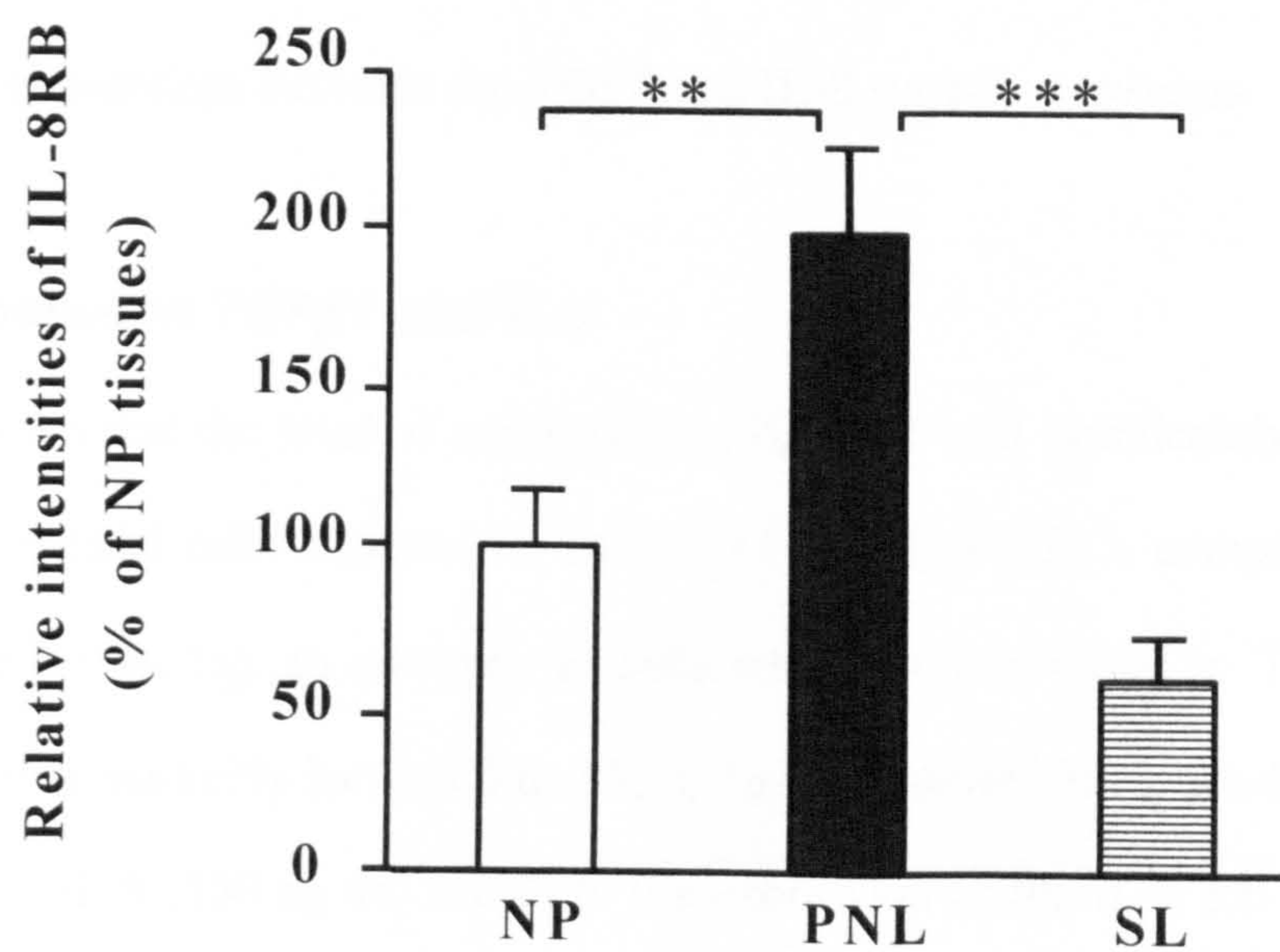
A. Western blots illustrating the expression of IL8-RB in tissue lysates prepared from non-pregnant (NP), pregnant non-labouring (P) and spontaneous labouring myometrium (SL). B illustrates analysis of 7 NP, 7 P and 7 SL tissue samples prepared from different patient groups. Relative IL-8 receptor levels in each tissue were determined by densitometry. The levels determined in each blot for the NP samples were arbitrarily set at 100% and the levels of expression in the P and SL samples expressed as a change relative to the NP level. The values shown are mean \pm SEM. **, $P < 0.01$; ***, $P < 0.001$.



A.



B.



4.2 IL-1 and IL-8 effects on TGF β 1 and T β RI expression

To investigate the effect of IL-1 and IL-8 on the expression on TGF β 1 and T β RI, confluent myometrial cells were incubated for 24 h with IL-1 or IL-8. Figure 4.3A shows that IL-8 at a concentration of 150 ng/ml significantly increased the expression of TGF β 1 in human myometrial cells from 21.4 ± 0.99 to 54.7 ± 2.0 pg/ μ g protein ($P < 0.001$). As shown in Figure 4.3B, the expression of T β RI was also induced by IL-8. The level of T β RI expression in control cells was set to 100 % and the level of T β RI expression increased to 152 ± 15 % compared to the control cells ($P < 0.05$). IL-1, at a dose chosen to be maximal for prostaglandin stimulation in human myometrial cells (10 ng/ml) (el Maradny *et al.* 1996a; Todd *et al.* 1996), significantly increased the total level of TGF β 1 to 29.5 ± 1.1 pg/ μ g protein ($P < 0.01$). At this concentration, IL-1 did not significantly influence the expression of T β RI compared to that found in the control cells. Because IL-8 affected both TGF β 1 and T β RI expression, further experiments were carried out in myometrial cells to explore the possible interactions between the TGF β and IL-8 signalling systems.

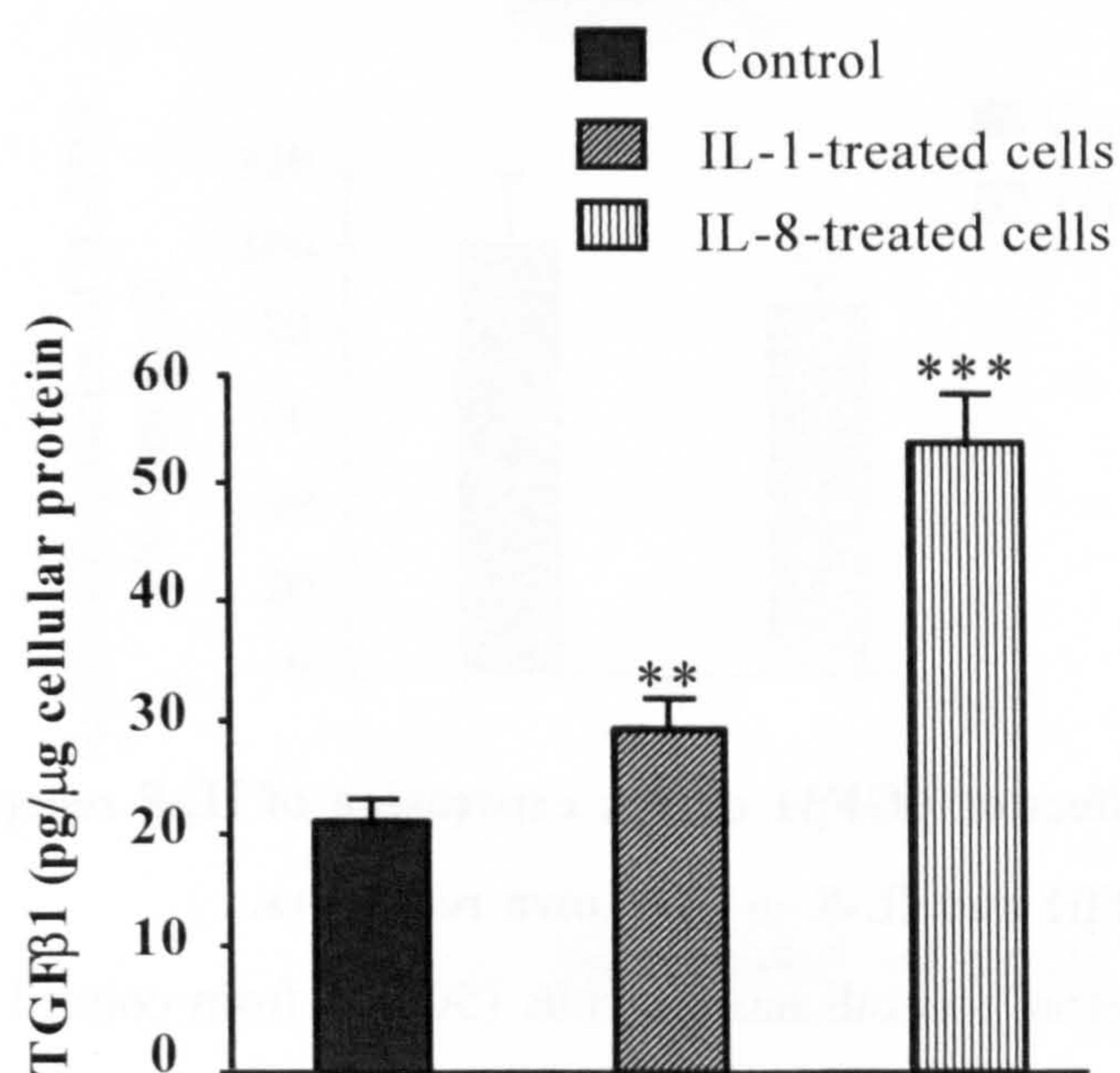
4.3 Interactions between TGF β 1 and IL-8

Figure 4.4A shows that the level of expression of IL-8 RB was significantly decreased to 84 ± 3 % in myometrial cells exposed to TGF β 1 (1 ng/ml) for 24 h compared to the control untreated cells (100 %). In contrast, in cells which were exposed to TGF β 1, the expression of T β RI was markedly increased to 158 ± 13 % as shown in Figure 4.4B. When cells were exposed to IL-8 (150 ng/ml) for 24 h, the expression of the IL-8 RB was found to be reduced and the level of expression was 84 ± 3 % as demonstrated in Figure 4.4C. Thus, there appears to be a complex relationship between these cytokines and the expression of cytokine receptors.

Figure 4.3 The effects of IL-1 and IL-8 on TGF β 1 and T β RI expression

A. Human myometrial smooth muscle cells were incubated with IL-1 (10 ng/ml) and IL-8 (150 ng/ml). The total concentration of TGF β 1 in the culture medium was measured after incubation for 24 h. Values are mean \pm SEM of four experiments from four different tissues assayed in duplicate ($n = 4$) and expressed as pg/ μ g protein. B. Myometrial smooth muscle cells (50 μ g) from control (C), IL-1 and IL-8 treated cells were separated in SDS-PAGE and subjected to Western blot analysis with specific antibody to T β RI. The results are expressed as means of triplicate measurements of T β RI expression in myometrial smooth muscle cells isolated from five different tissue samples. Densitometry data are expressed as a percentage of control. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ as compared to the control.

A.



B.

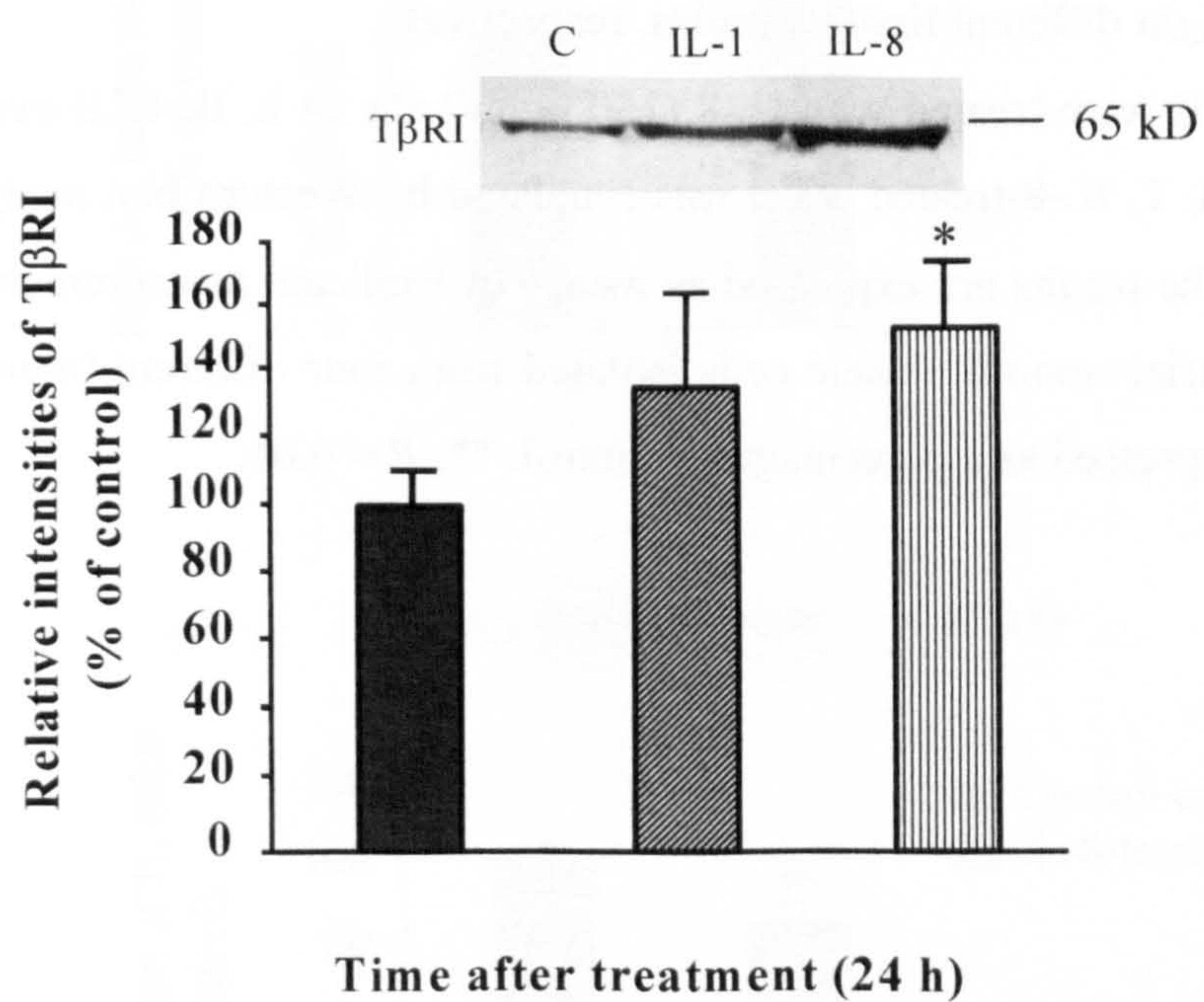
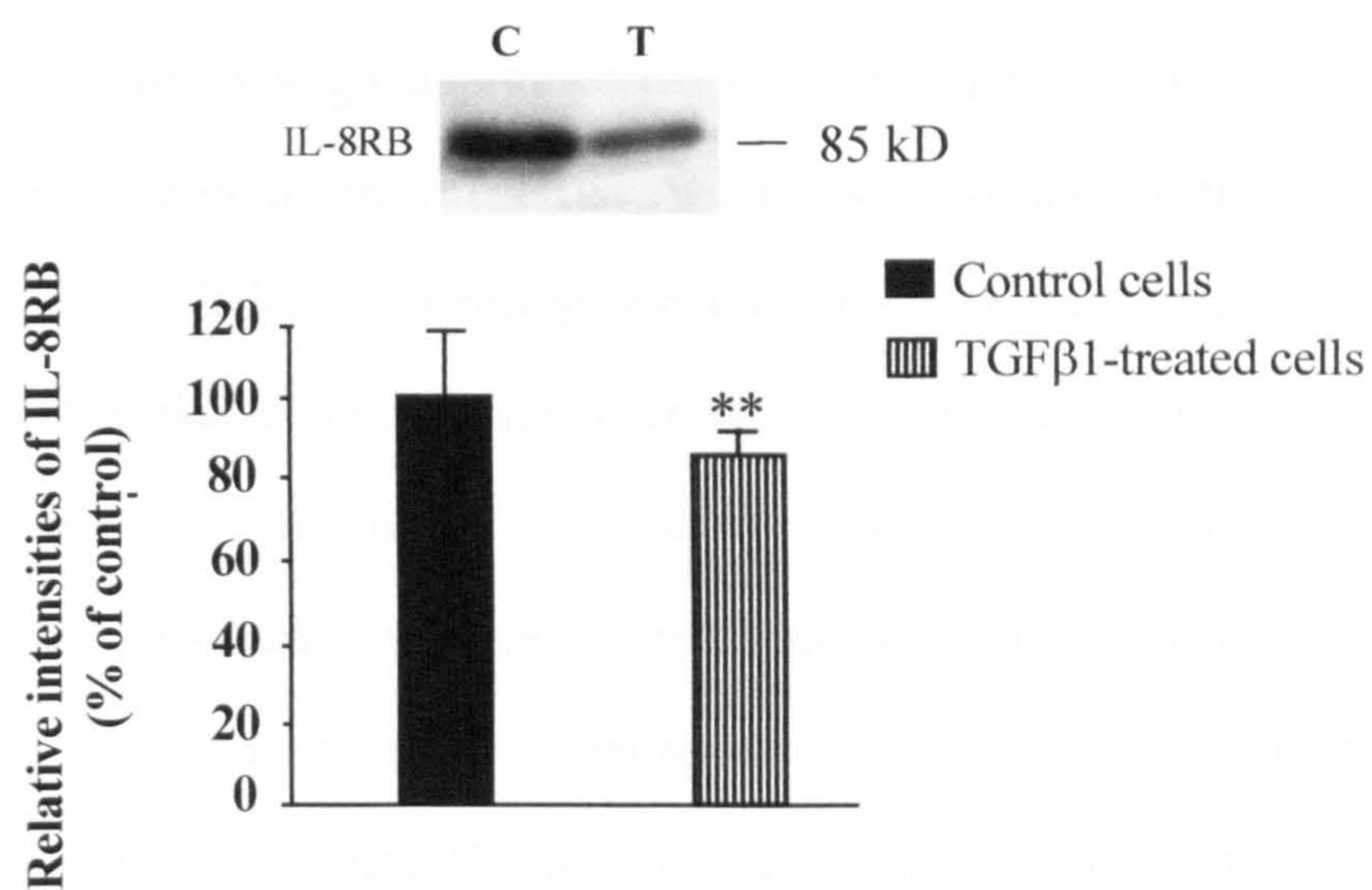


Figure 4.4 The effect of TGF β 1 on the expression of IL-8 receptor type B (IL-8RB) and the effects of TGF β 1 and IL-8 on their own receptors.

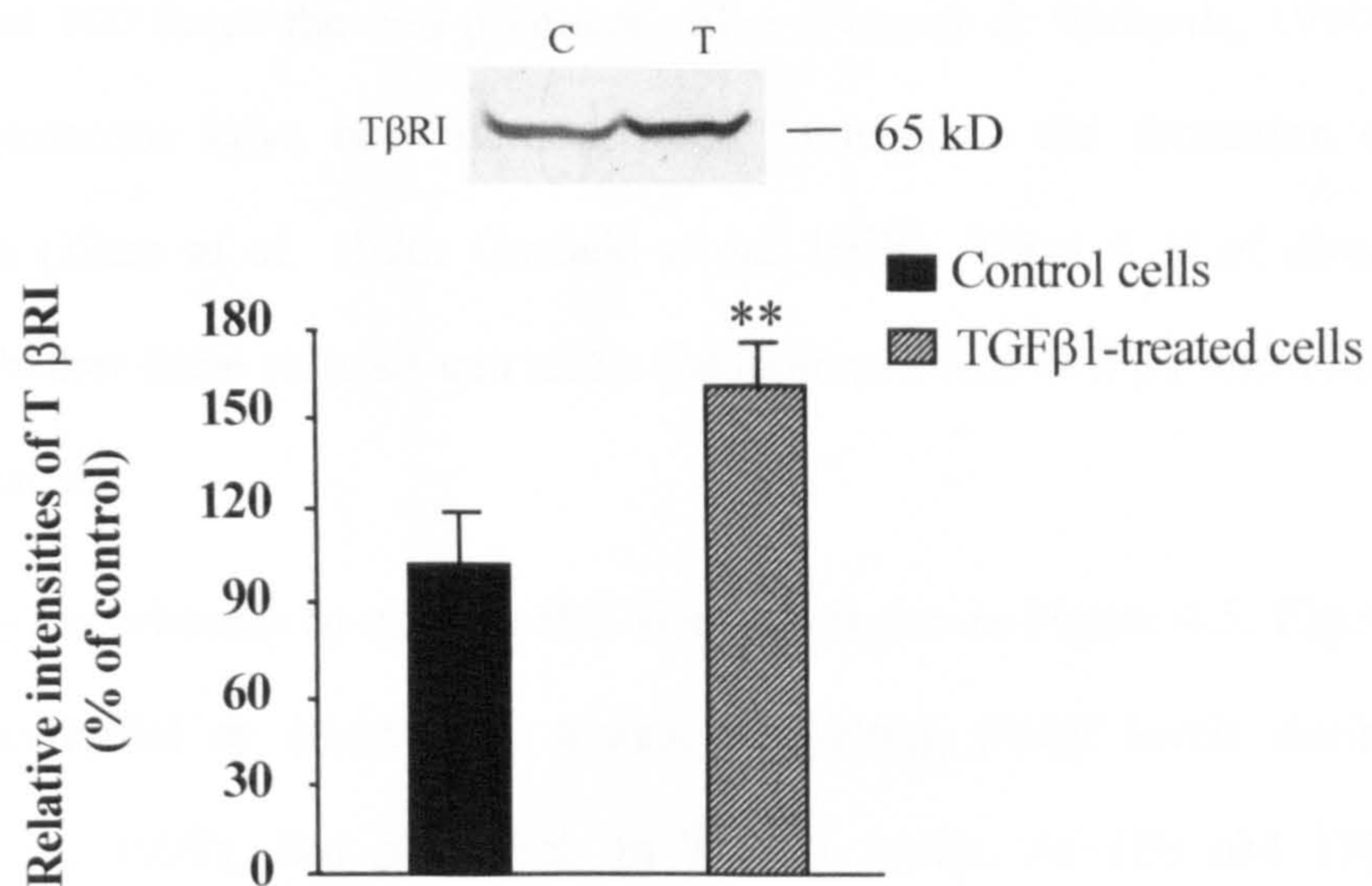
A. and B. Myometrial smooth muscle cells (50 μ g) from control (C) and cells treated with TGF β 1, 1ng/ml (T) for 24 h were separated in SDS-PAGE and subjected to Western blot analysis with antibodies to IL-8RB and T β RI. The results are expressed as means of triplicate measurements of IL-RB and T β RI expression in myometrial smooth muscle cells isolated from four and eight different tissue samples, respectively.

C. The cells were treated with IL-8 (150 ng/ml) for 24 h. IL-8RB expression from these cells (C, control; T, IL-8-treated cells) was evaluated by Western blot analysis of cell homogenates (50 μ g). The results are expressed as means of triplicate measurements of IL-8RB expression in myometrial smooth muscle cells isolated from four different tissue samples. Densitometry data are expressed as a percentage of control. **, $P < 0.01$.

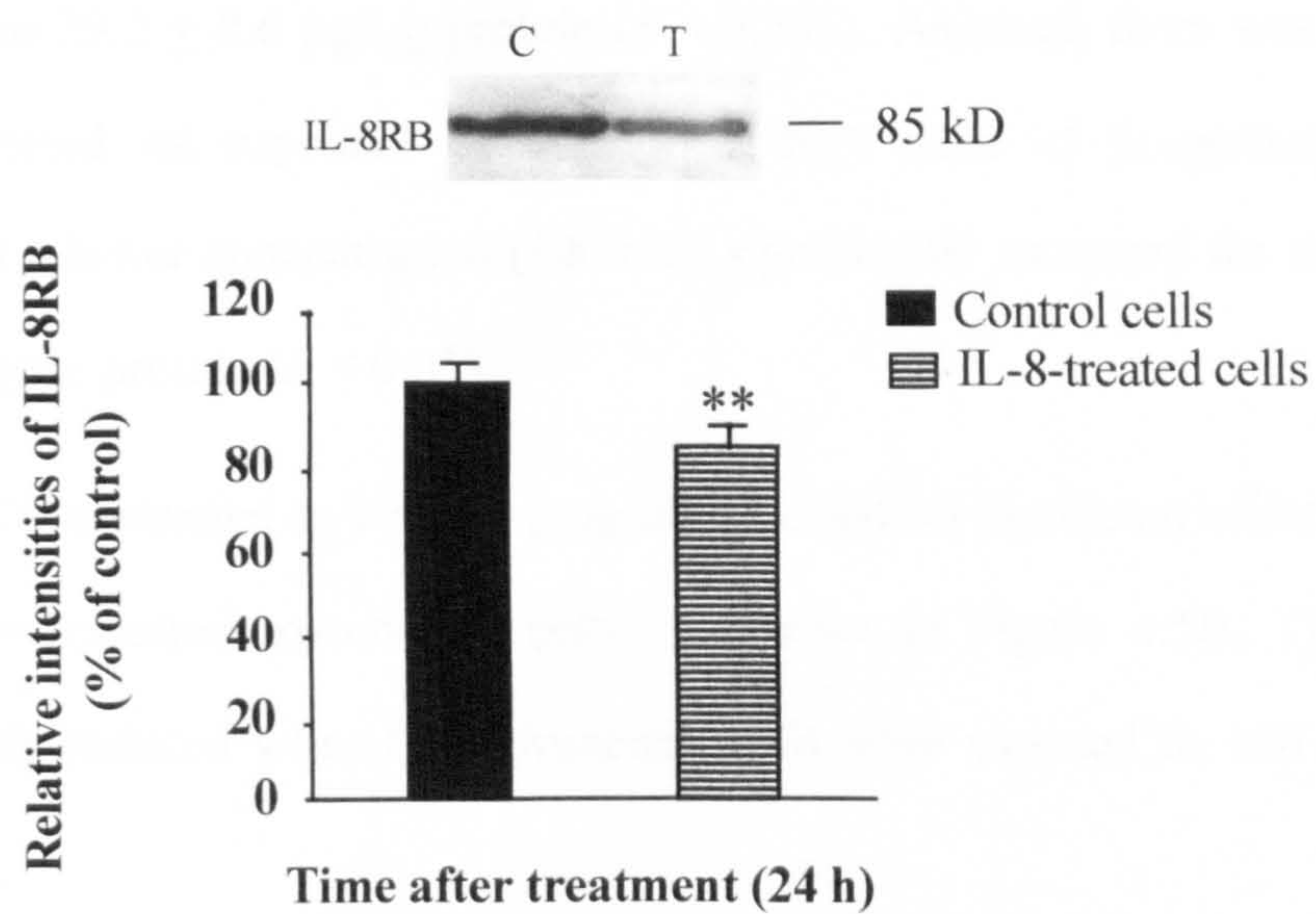
A.



B.



C.



4.4 Oestrogen and progesterone effects on $TGF\beta 1$ and $T\beta RI$ expression

Oestrogen and progesterone undergo a progressive rise throughout pregnancy and changes in the level of these steroids have been shown to be associated with human parturition (Liu & Rebar, 1999). It has been recognised for some time that progesterone acts as a myometrial relaxant, which maintains quiescence during pregnancy (Csapo, 1977). Oestrogen, in contrast, increases myometrial contractility and enhances myometrial sensitivity to other substances involved in the mechanism of myometrial contraction (Lye, 1996). In the non-pregnant state, the main oestrogen secreted by the developing follicle during the menstrual cycle is 17β -oestradiol. During pregnancy, the concentrations of this hormone increase to about 100 times the non-pregnant value (Pocock & Richards, 1999). 17β -oestradiol and progesterone have been demonstrated to regulate the formation of myometrial gap junctions (Zhao *et al.* 1996; Garfield *et al.* 1999). Thus, it is of direct relevance to determine whether these steroids can affect the expression of $TGF\beta 1$ and $T\beta RI$ in myometrial smooth muscle.

Data from a series of experiments to explore this idea are shown in Figure 4.5. Figure 4.5A shows that 17β -oestradiol at 10 nM, the lowest circulating blood levels during pregnancy (Andersen *et al.* 1993), had no effect on $TGF\beta 1$ levels. At 100 nM 17β -oestradiol, the concentration of $TGF\beta 1$ was significantly increased from the control level of 20.7 ± 1.3 pg/ μ g protein to 29.2 ± 0.6 pg/ μ g protein ($P < 0.001$). Although there was no significant difference detected on exposure of cells to a high dose of progesterone (200 nM), progesterone at a lower concentration (100 nM) significantly increased the level of $TGF\beta 1$ to 26.2 ± 1.3 pg/ μ g protein ($P < 0.01$).

Exposure to 10 nM 17β -oestradiol or 100 nM progesterone had no significant effect on the expression of $T\beta RI$ in cultured myometrial cells. As shown in Figure 4.5B, $T\beta RI$ expression was significantly reduced when the myometrial cells were exposed to 100 nM

17 β -oestradiol or 200 nM progesterone. The percentage reductions following 17 β -oestradiol and progesterone exposure were 61.4 ± 10.5 and 47.8 ± 9.5 , respectively.

In vivo it is likely that there may be changes in both steroid hormone levels as well as cytokine concentrations. From Figure 4.3, it was shown that IL-1 and IL-8 alone significantly increased the release of TGF β 1 into the culture medium. Figure 4.6 illustrates data examining the combined effects of IL-1, IL-8 and 17 β -oestradiol. The results show that 17 β -oestradiol at 100 nM reduced the stimulatory effect of IL-8 on the release of TGF β 1. When IL-1 and IL-8 were added simultaneously there was an increase in TGF β 1 release compared to control but this was significantly lower than the stimulation produced by IL-8 alone (32.8 ± 1.7 compared to 54.7 ± 2.0 pg/ μ g protein). Thus, it appears that 17 β -oestradiol and IL-1 can inhibit the stimulatory effect of IL-8 *in vitro*. The consequences of these complex interactions *in vivo* have yet to be explored.

Figure 4.5 The effects of 17 β -oestradiol and progesterone on TGF β 1 and TGF β receptor type I (T β RI) expression

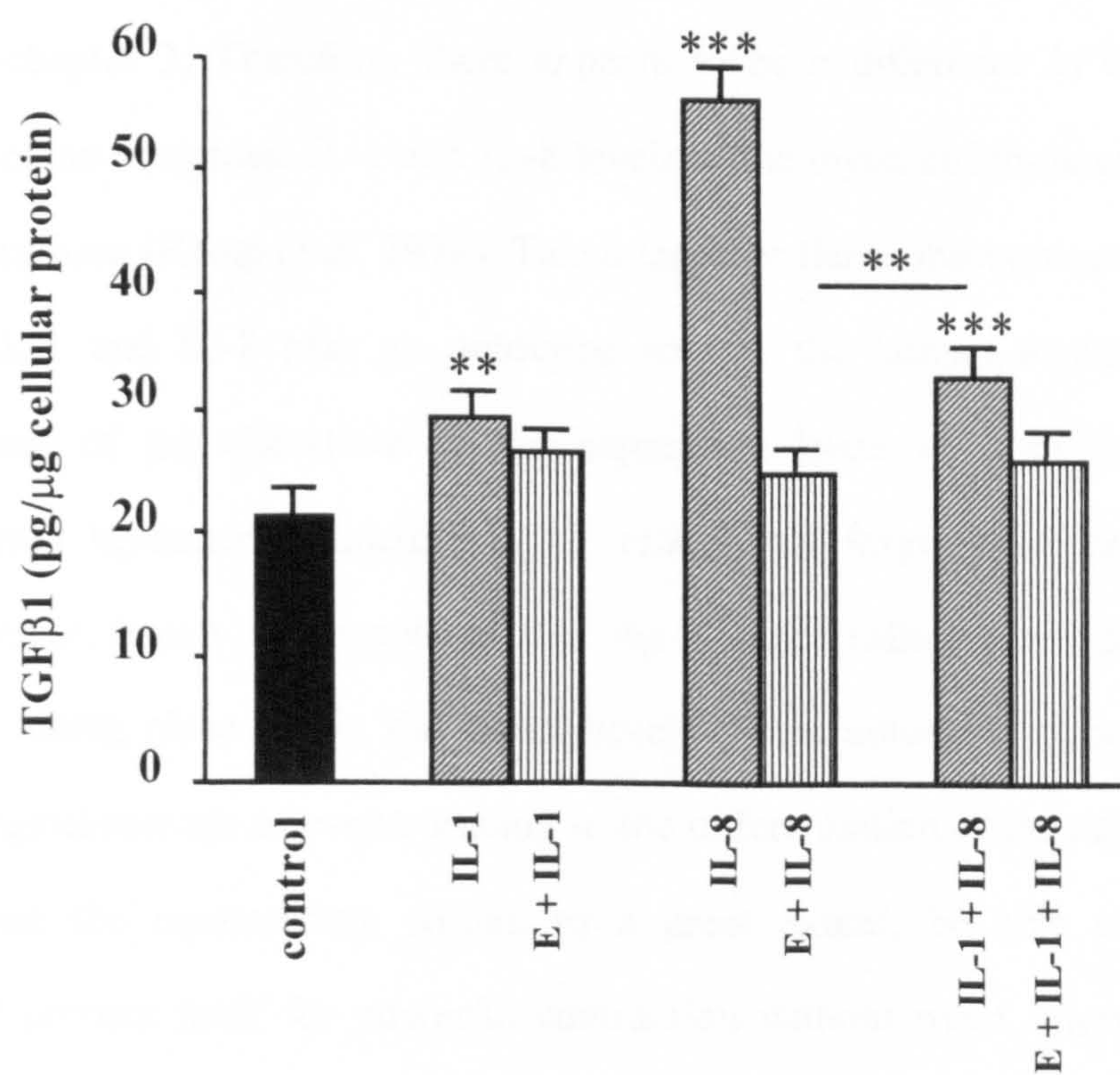
A. Human myometrial smooth muscle cells were incubated with 17 β -oestradiol (10 nM and 100 nM) and progesterone (100 nM and 200 nM). The total concentration of TGF β 1 in the culture medium was measured after incubation for 24 h. Values are mean \pm SEM of four experiments from four different tissue samples assayed in duplicate (n = 4) and expressed as pg/ μ g protein.

B. Myometrial smooth muscle cells (50 μ g) from control (C) and treated cells [17 β -oestradiol (E10 and E100) and progesterone (P100 and P200)] were separated in SDS-PAGE and subjected to Western blot analysis with a specific antibody to T β RI. The results are expressed as means of triplicate measurements of T β RI expression in myometrial smooth muscle cells isolated from five different tissue samples. Densitometry data are expressed as a percentage of control. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 4.6. The effects of 17 β -oestradiol, IL-1, IL-8 and their combinations on TGF β 1 production

Human myometrial smooth muscle cells were incubated with 17 β -oestradiol (100 nM), IL-1 (10 ng/ml), IL-8 (150 ng/ml) and their combinations for 24 h. The total concentration of TGF β 1 in the culture medium were measured. Values are mean \pm SEM of four experiments from four different tissue samples assayed in duplicate ($n = 4$) and expressed as pg/ μ g protein. **, $P < 0.01$; ***, $P < 0.001$ (there is significantly different $P < 0.001$ in each group as compared to the IL-8-treated cells).





4.5 Discussion

The observations made in this study suggest that the receptor levels for IL-1 and IL-8 in the myometrium change with pregnancy. These results support previous observations that IL-8 receptors are present in the human myometrium at term before the initiation of labour (el Maradny *et al.* 1996b). It may be important to note that the patterns of change in receptor expression are different for both receptors. IL-1 receptor expression is greatest in the non-pregnant tissue, lower in the pregnant non-labouring tissue and lowest in the spontaneous labouring tissue. In contrast, IL-8 receptor expression is maximal in pregnant non-labouring myometrium and falls in labouring myometrium. The changes in the expression of IL-8RB are similar to the patterns of change for TGF β receptors as previously demonstrated in chapter 3. Therefore, there appears to be a difference in the timing of expression of cytokine receptors. IL-1 and IL-8 levels in the myometrium have been found to increase at parturition (Kemp *et al.* 1998). Taken together these observations point to the possibility that IL-1 and IL-8 play an autocrine role in the human myometrium. The particular relevance of the alterations in the expression levels of both cytokines and receptors to overall myometrial functioning at term is not known and has yet to be established. However, it may be speculated that the changes reflect a complex series of autocrine signals taking place within the myometrium. These autocrine signals may form part of a physiological cascade of events leading to the differentiation of the myometrium. If this were the case the myometrium would, to a great extent, be able to control its development and prepare itself for powerful contraction without overt intervention from fetal or other maternal systems.

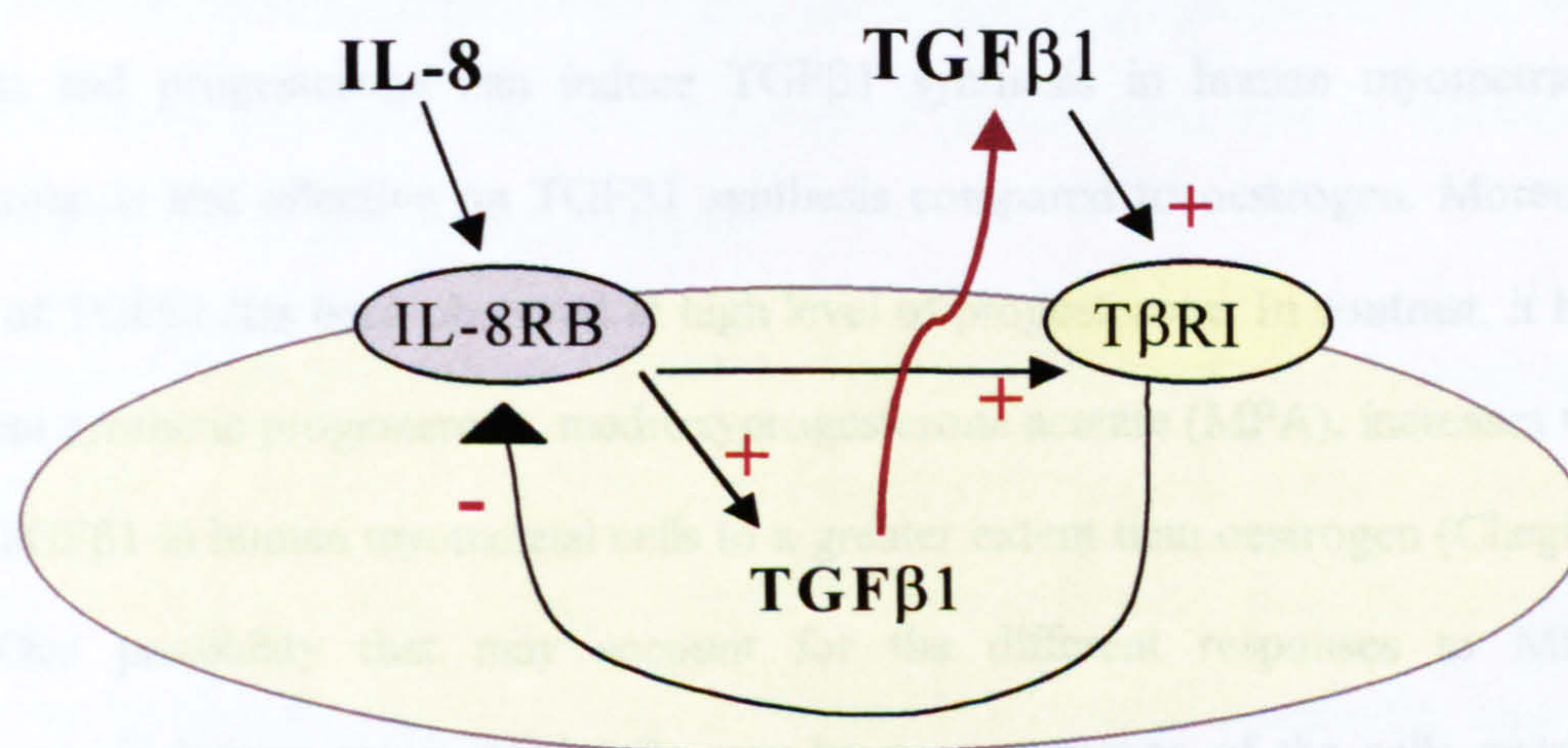
There is evidence for the involvement of cytokines in the modulation of uterine function in pre-mature labour (see Mazor *et al.* 1995). The final result of the cytokine activation is an increase in the local concentration of prostaglandin, which in turn has complex actions on the myometrium and cervix (Mitchell *et al.* 1991a; Norwitz *et al.* 1992; Pollard *et al.* 1993). The resulting strong myometrial contractions, along with cervical softening, can initiate

premature labour and subsequent delivery. If the idea of an autocrine physiological signalling cascade is correct, then it is not surprising that exogenous cytokines originating from leukocytes activated by infection can prematurely activate this cascade with a consequence of premature delivery.

It is therefore important to understand the complexities of these autocrine signalling events and the interactions between components, which are termed signalling associated proteins (SAPs), in the cascade. As it is not possible to interfere with the pregnant myometrium *in vivo*, an alternative approach is to use human myometrial cells *in vitro*. The findings in this study indicate that both IL-1 and IL-8 increase TGF β 1 secretion in human myometrial smooth muscle cells in culture. The mechanisms involved are unknown. There is evidence to demonstrate that binding of IL-8 to IL-8 receptors activates extracellularly responsive kinase (ERK), resulting in an increase of c-fos mRNA (Shyamala & Khoja 1998). The human TGF β 1 promoter region also contains activator protein-1 (AP-1) sites which are composed of c-fos and c-jun genes (Kim *et al.* 1990; Scotto *et al.* 1990), thus, IL-8 may stimulate TGF β 1 production through activating AP-1 site on the TGF β 1 promoter. Post-transcriptional and Post-translational regulation also affects the production of some cytokines (Hamblin, 1993). The increase in TGF β 1 levels in the myometrial smooth muscle cells by IL-1 and IL-8 may result from such regulations. It is also possible that the action of IL-1 on TGF β 1 may be mediated indirectly via other agents. For example it is known that IL-1 can increase the production of PG (Todds *et al.* 1996). PG could therefore affect the level of TGF β 1 production. This possibility was not examined in this thesis.

A synergism between these two cytokines has not previously been observed and it is likely that IL-1 can inhibit the stimulatory effect of IL-8 on TGF β 1 secretion from the myometrial smooth muscle cells (see Figure 4.6). Thus, it can be suggested that higher levels of TGF β 1 may be generated in the human myometrial cells *in vivo* if IL-1 and IL-8 are produced and presented at different times, and in a specific sequence.

IL-1 and IL-8 also increase the level of T β RI expression although IL-8 appears to be more effective than IL-1. Thus, under these conditions, both cytokines affect the steady state level of both TGF β 1 and its receptor. It has been found in this study that TGF β 1 induces up regulation of its receptor (see Figure 4.4B) and inhibits the expression of IL-8RB (see Figure 4.4A) in the myometrial cells *in vitro*. The up regulation of T β RI induced by TGF β 1 has been reported recently in cultured pancreatic epithelial cells in rat (Menke *et al.* 1999). The level of IL-8RB is down regulated by IL-8 (see Figure 4.4C). This IL-8 induced down regulation of its own receptor has also been reported in other cell types (Chuntharapai & Kim, 1995; Ray & Samanta, 1996). One possible interpretation of these data would be that IL-8 would be activated at an earlier stage such that IL-8 would stimulate TGF β 1 production and T β RI expression. TGF β would then aid increasing its receptor expression providing a positive feedback and contributing to the increase of TGF β -induced cellular responses. TGF β would also down regulate the IL-8 receptor, thus, decreasing its effectiveness. In this way one cytokine signal would decrease whilst another would increase in importance. A Model representing the interaction between the TGF β 1 and IL-8 systems is shown below.



In human endometrial cells, TGF β 1 acts to potentiate the effect of IL-1 α to increase the level of IL-8 expression (Arici *et al.* 1996c). There is evidence that IL-1 and TNF α can regulate the level of IL-8 production in human endometrial cells in culture, human chorion and decidual cells (Dudley *et al.* 1993; Arici *et al.* 1996). It is reasonable to hypothesise that the levels of IL-8 in human myometrial smooth muscle cells can be increased by IL-1 and TNF α . The involvement of IL-8 in the process of cervical dilation has been well established (Chwalisz *et al.* 1994; Osmer *et al.* 1995) but the crucial role of IL-8 in the myometrium is not well understood. However, by using the *in vitro* model, it can be suggested that interactions between IL-1, IL-8 and TGF β 1 occurs in the human myometrium at term before labour begins. The sequence of events in the myometrium may be initiated when IL-1 induces IL-8 followed by IL-8 induction of TGF β 1 and its receptor and finally TGF β 1 up-regulation of the expression of CAP genes such as RyR2 (Awad *et al.* 1997). TGF β 1 can increase the synthesis of COX2 and endothelin-1 in other cell types (Xu *et al.* 1997; McKenna *et al.* 1998). Thus, there appears to be a complex interplay between cytokines, which can act to promote the expression of SAPs and the expression of CAPs.

The levels of expression of cytokines can also be regulated by sex steroids. The observations in this study confirm that the expression of TGF β 1 and the T β RI appear to be regulated by oestrogen and progesterone but in a different way. This study shows that oestrogen and progesterone can induce TGF β 1 synthesis in human myometrial cells. Progesterone is less effective on TGF β 1 synthesis compared to oestrogen. Moreover, no increase of TGF β 1 has been observed at high level of progesterone. In contrast, it has been shown that synthetic progesterone, medroxyprogesterone acetate (MPA), increases the total level of TGF β 1 in human myometrial cells to a greater extent than oestrogen (Chegini *et al.* 1996). One possibility that may account for the different responses to MPA and progesterone in human myometrial cells may be a consequence of the cells metabolising progesterone to a form that does not bind to the progesterone receptor (Dombroski & MacDonald, 1993).

It has been shown that there is no oestrogen response element (ERE) in the TGF β 1 promoter (Kim *et al.* 1989). However, incomplete estrogen response element-like sequences have been reported in the TGF β 1 gene (as cited by Hering *et al.* 1995). It might be that the increase in TGF β 1 production by oestrogen is mediated by ERE-like elements. Oestrogen is known to up-regulate the expression of *c-fos* and *c-jun* genes (Loose-Mitchell *et al.* 1988; Weisz *et al.* 1990). Thus, an alternative explanation could be the stimulation of TGF β 1 via the activation of *c-fos* and *c-jun*, which will bind to AP-1 site on the TGF β 1 promoter.

The present study has shown that high doses of oestrogen and cytokines alone and in combination were more effective in increasing the total level of TGF β 1 than was progesterone. In this case the effect of oestrogen, IL-1 and IL-8 may act separately in the myometrium or in concert to overcome the effect of a high level of progesterone on TGF β 1 production *in vivo*.

In humans, contrast to many animals at parturition, the plasma concentrations of oestrogen and progesterone do not fall prior to the onset of labour (Batra & Bengtsson, 1978; Fuchs & Fuchs, 1984). There is evidence to suggest that TGF β 1 may act like an anti-progestin (Casey & MacDonald, 1996a, 1996b). If this is correct then a high level of TGF β 1 in the myometrium at term may inhibit the actions of progesterone. Thus, inhibition of progesterone action, rather than a withdrawal of progesterone, may be involved in the changes seen in uterine activity before labour.

Oestrogen and progesterone also affect the level of T β RI expression. In contrast to the stimulatory effect of oestrogen on TGF β 1, oestrogen and progesterone act to decrease the expression of T β RI in human myometrial cells. This is in keeping with the observation *in vivo*, that the level of expression of T β RI is reduced in spontaneous labouring myometrium at a time when the plasma levels of both steroids are high. Thus, the expression of T β RI and TGF β 1 are regulated in the opposite way by sex hormones in human myometrium. These

data suggest an interaction between endocrine and autocrine systems in the myometrial cells. It can be concluded from these overall observations that a complex and fine balance between cytokine and endocrine networks underlies the physiological process regulating the preparation of the human myometrium for term.

Chapter 5

Myometrial COX-2 expression and identification of synergistic and sequential actions of cytokines on T β RI and COX-2 expression in human myometrial cells *in vitro*

There is substantial evidence that prostagandins (PG), which are synthesised from arachidonic acid, play a role in the initiation of parturition and the onset of pre-term labour (Kelly, 1994). Cyclooxygenase (COX) or prostaglandin H synthase (PGHS) is believed to be involved in the initial reaction of PG synthesis (Rosen *et al.* 1989). It is generally accepted that myometrium is the target of PG action rather than the site of PG production (Moore *et al.* 1999). However using immunohistochemical technique, this enzyme has been found in the myometrium and localised in the smooth muscle (Zuo *et al.* 1994). This is indicative of the biosynthesis of PG in the myometrium.

COX-1 is the constitutive form, whereas COX-2 is a regulated form of the enzyme (Hla *et al.* 1986; Morita *et al.* 1995). *In situ* hybridisation and immunocytochemistry has demonstrated that the expression of COX-1 mRNA and protein were lower at term compared to pre-term whilst the levels of COX-2 were increased at term. Moreover, COX-2 levels were lower during labour compared to that of non-labouring tissue (Zuo *et al.* 1994). In contrast to this study, Moore *et al.* (1999) have reported that both COX-1 and COX-2 mRNA did not change significantly during gestation or at parturition, although the expression of COX-1 mRNA appeared higher at term. Recently, it has been shown that levels of COX-2 protein were unaffected by the onset of labour (Sparey *et al.* 1999)

As previous reports on the expression of COX-2 in the myometrium are conflicting, an attempt to measure the level of COX-2 was performed in the human myometrium with a view to addressing the question, "Does the level of COX-2 protein change with gestational age or with labour? ". The experiment was then conducted on the myometrial tissue lysates

from pre-term non-labouring, pregnant term non-labouring and spontaneous labouring women.

5.1 COX-2 expression

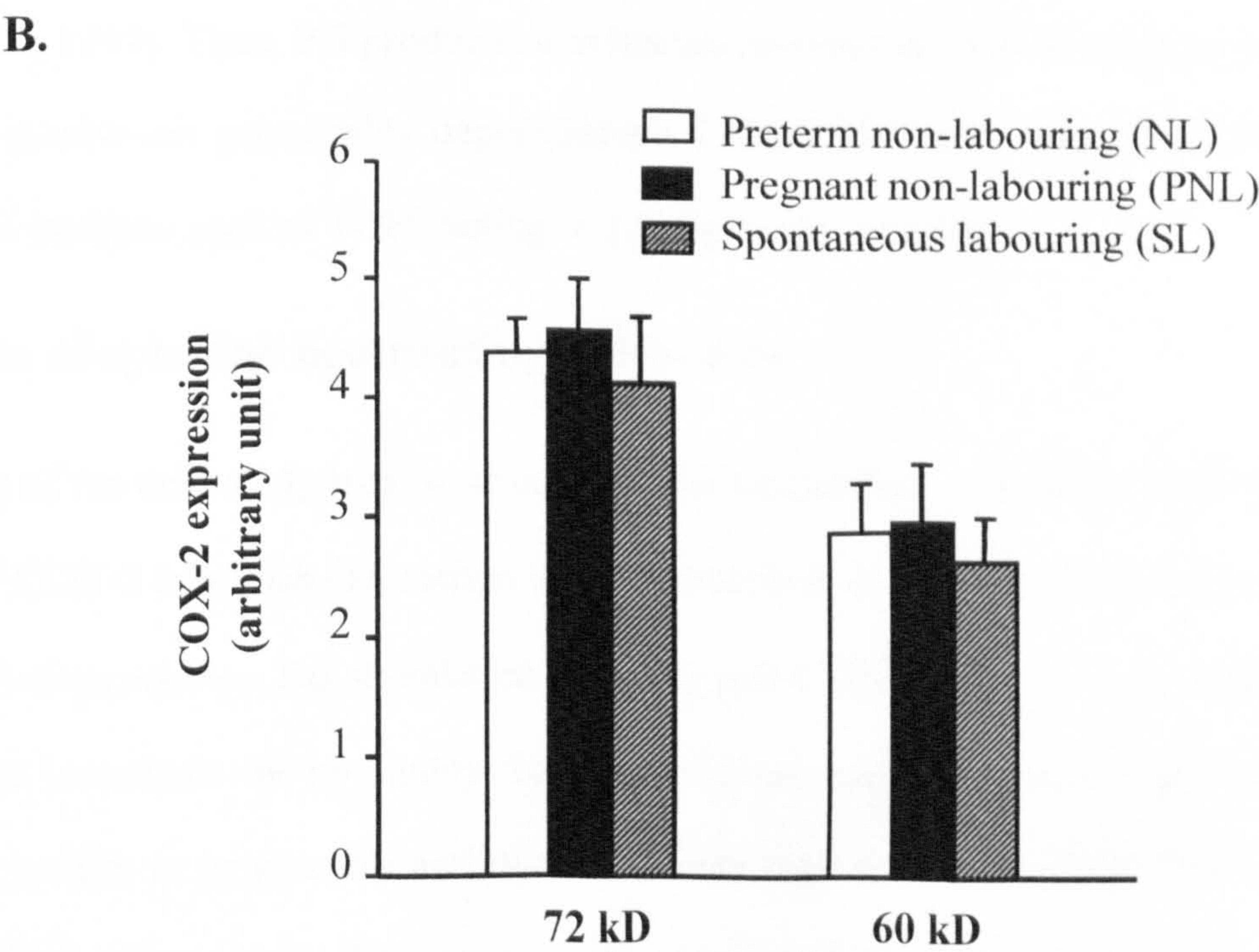
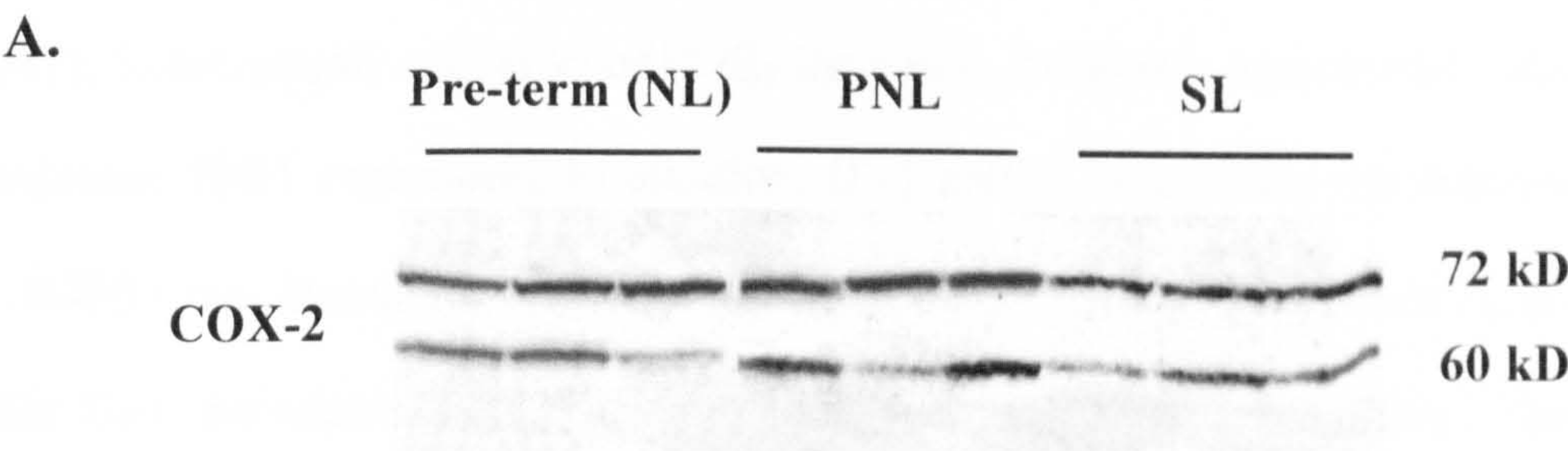
Using Western blot analysis, COX-2 was found in the human myometrium in all tissue groups. As demonstrated in Figure 5.1A, myometrial lysates from three different samples in each group contained two bands of COX-2 with the molecular masses of 56-60 kD and 72 kD. This finding is consistent with previous observations (Zuo *et al.* 1994). Figure 5.1B shows accumulative data from a densitometric analysis, obtained from five pre-term, seven term non-labouring and five term labouring myometrial samples. It was found that there were no significant differences in both forms of COX-2 expression among these groups. This observation was different from the COX-2 protein level reported by Zuo *et al.* (1994) but similar to COX-2 mRNA results as demonstrated recently by Moore *et al.* (1999) and COX-2 protein reported by Sparey *et al.* (1999).

Although the myometrial COX-2 level does not change during pregnancy and parturition, particular attention was made to COX-2 as its expression can be regulated by cytokines in a variety of cell types (Mitchell *et al.* 1993; Xu *et al.* 1997; Diaz *et al.* 1998). In response to IL-1 and TNF α , the levels of PG in human amnion and myometrial cells increase. This is presumably by the induction of expression of COX-2 (Todd *et al.* 1996). This pathway is known to be involved in the initiation of pre-term labour. It can be proposed that alterations in the levels of this protein and others not involved directly in contraction such as oxytocin and TGF β receptors are associated with an increase in myometrial activity. By regulating the synthesis of these proteins, which have been described as Signalling Associated Proteins (SAPs), the myometrial cells can regulate their ability to respond to stimuli and their excitability and contractility.

Figure 5.1 The expression of COX-2 in pregnant myometrium at 31-35 and 38-40 weeks of gestation

A. Western blots illustrating the expression of COX-2 in lysates prepared from myometrial tissues from pre-term non-labouring (31-35 weeks), pregnant non-labouring (PNL, 38-40 weeks) and spontaneous labouring myometrium (SL).

B illustrates analysis of COX-2 levels in the myometrium. The levels of COX-2 expression were determined by densitometry. The values shown are mean \pm SEM of five samples from pre-term, seven samples from PNL and five samples from SL myometrium.



Cytokines may play a role in regulating the synthesis of SAPs and CAPs. Cytokine stimulation of cellular events is likely to be complex. One cytokine may increase the synthesis of a second cytokine, which triggers gene activation. An example of this type of interaction has been reported in human osteoblasts (Xu *et al.* 1997). TNF α increases prostaglandin E₂ (PGE₂) production. However, experiments using an IL-1 neutralising antibody, shows that TNF α acts indirectly by stimulating the autocrine production of IL-1 (Xu *et al.* 1997). Subsequently, the level of PGE₂ increases. In human myometrial cells, IL-1 and IL-8 increase T β RI expression. In addition, IL-1 and IL-8 increase the synthesis of TGF β 1 and TGF β 1 can increase the expression of T β RI (see chapter 4). This raises the possibility that IL-1 stimulates TGF β 1, which then activates T β RI expression. There is evidence to suggest that TGF β enhances the stimulatory effect of IL-1 on PGE₂ production and the mechanism appears to be mediated primarily by the regulation of COX-2 expression (Pilbeam *et al.* 1997). Thus, PG production in human myometrial cells in response to IL-1, acting by a mechanism presumably dependent on COX-2 synthesis, may also involve an intermediate cytokines such as TGF β acting in a 'long range' manner.

The use of cytokine neutralising antibodies

The aim of the next study was to investigate the mechanisms regulating the expression of T β RI and COX-2 in human myometrial smooth muscle cells and to explore the possibility of cytokine interactions on the stimulation of T β RI and COX-2. Several approaches were considered to investigate the possibility that one cytokine may influence the production of the second cytokine or its receptor and that the effects seen as a result of the first cytokine are mediated via the second. The approaches considered were a) the use of cytokine neutralising antibodies or b) the use of antisense oligonucleotides. In this study, for reason of time, the initial approach was to use neutralising antibodies and Western blotting to measure the levels of T β RI and COX-2 expression. Cytokine neutralising antibodies were used to neutralise the effects of endogenous cytokines released following exogenous

cytokine stimulation. Chegini *et al* (1996) has demonstrated that myometrial cells in culture can synthesise and release active TGF β 1 at concentration of approximately of 0.7 ng/ml. The concentration of TGF β 1 neutralising antibody used for this study has been calculated from the guideline provided by the manufacturer (R&D Systems). It has been shown that approximately 1 μ g/ml of the antibody neutralised almost 100 % of the bioactivity due to 0.25 ng/ml of TGF β 1. Thus, 3 μ g/ml of TGF β 1 neutralising antibody has been used in this investigation. The optimal concentration of the neutralising antibody required would depend on the cytokine concentrations, cell types, cell numbers and growth conditions. The optimisation was not done in this experiment since the preliminary condition chosen demonstrated positive results. Additional work is, however, required to characterise this effect. Furthermore, the neutralising antibodies were used with the assumption that the specificity and efficacy were that reported by the manufacturer. No independent validation was undertaken prior to the experiments. This may lead to a potential problem interpreting data when no effect of neutralising antibody was found. However, where a clear change was noted it would seem reasonable to assume that the neutralising antibodies were inhibiting the proteins.

5.2 IL-1 and IL-8 stimulation of T β RI receptor expression

Figure 5.2A shows an example of a Western blot examining the expression of T β RI in cultured human myometrial cells. Lane 1 shows basal levels of T β RI expression in non-treated cells. Lanes 2 and 4 show the level of T β RI in cells following 24 h treatment with IL-1 and IL-8 respectively. The expression of T β RI was increased by exposure to both cytokines. We then analysed the levels of T β RI expression from four sets of measurements made on cells prepared from four different samples as shown in Figure 5.2B. In order to compare measurements between blots, the level of T β RI expression in control cells from each blot was set as 100 %. The pooled data showed a significant increase in T β RI levels after IL-1 (lane 1) and IL-8 (lane 4) stimulation. The mean values after stimulation were

145.75 \pm 10 % and 153.25 \pm 11 % for IL-1 and IL-8, respectively ($P < 0.01$). Lanes 3 and 6 show the levels of expression of T β RI in cells pre-treated with TGF β 1 neutralising antibody for 3 h prior to stimulation for 24 h with IL-1 and IL-8. Compared to the control cells, the levels of T β RI expression were 107.5 \pm 7.5 % and 102.33 \pm 14 %, respectively. These values are not statistically different from the control level but are significantly reduced compared to the levels found in the IL-1 and IL-8 treated-cells alone ($P < 0.01$ and $P < 0.05$, respectively). These data suggest that the stimulatory effects of IL-1 and IL-8 on T β RI expression are indirect and due possibly to their ability to increase myometrial cell TGF β 1 production, which can then act in an autocrine manner. Lane 5 shows data from cells pre-treated with IL-1 neutralising antibody prior to treatment with IL-8. The mean level of expression was found to be 153 \pm 19 % and significantly greater than basal expression ($P < 0.01$) but not different from the IL-8 stimulated cells. This observation shows that IL-8 regulation of T β RI does not involve any co-operative with IL-1.

Figure 5.2A shows two positively staining bands for the T β RI. There was a weak band at more than 65kD and more pronounced band at 60-65kD. The weak bands also appear to vary with the treatment of the cells being more pronounced in IL-8 treated cells (lane 4) and IL-1_(NA) + IL-8-treated cells (lane 5). The nature of the weaker bands is not known. However, such a small increase in molecular weight may reflect a biochemical modification of the receptors such as glycosylation or phosphorylation. A further characterisation of these weak bands was not carried out in the present study.

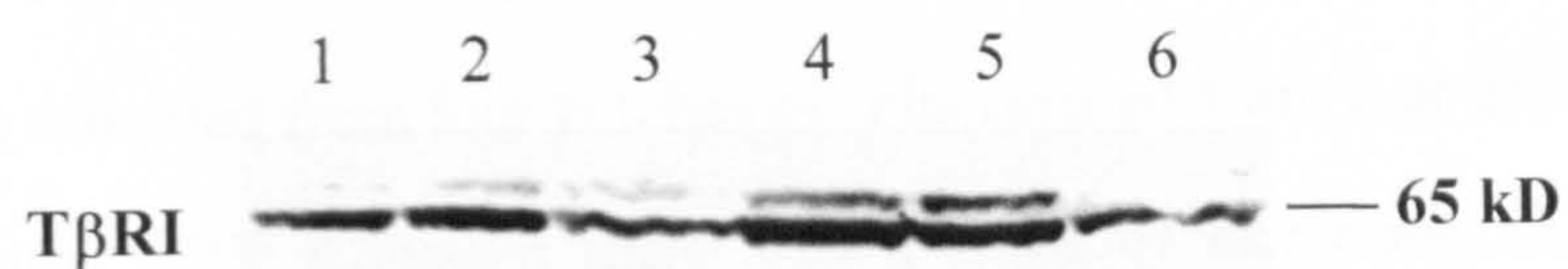
Figure 5.2 The effects of IL-1 and IL-8 on T β RI expression in the presence and absence of TGF β 1 and IL-1 neutralising antibody

A. Western blots illustrating the expression of T β RI. The individual lanes show: 1. control level of T β RI expression in untreated cells; 2. cells treated with IL-1; 3. cells treated with IL-1 and neutralising antibody to TGF β 1; 4. cells treated with IL-8; 5. cells treated with IL-8 and neutralising antibody to IL-1; 6. cells treated with IL-8 and neutralising antibody to TGF β 1.

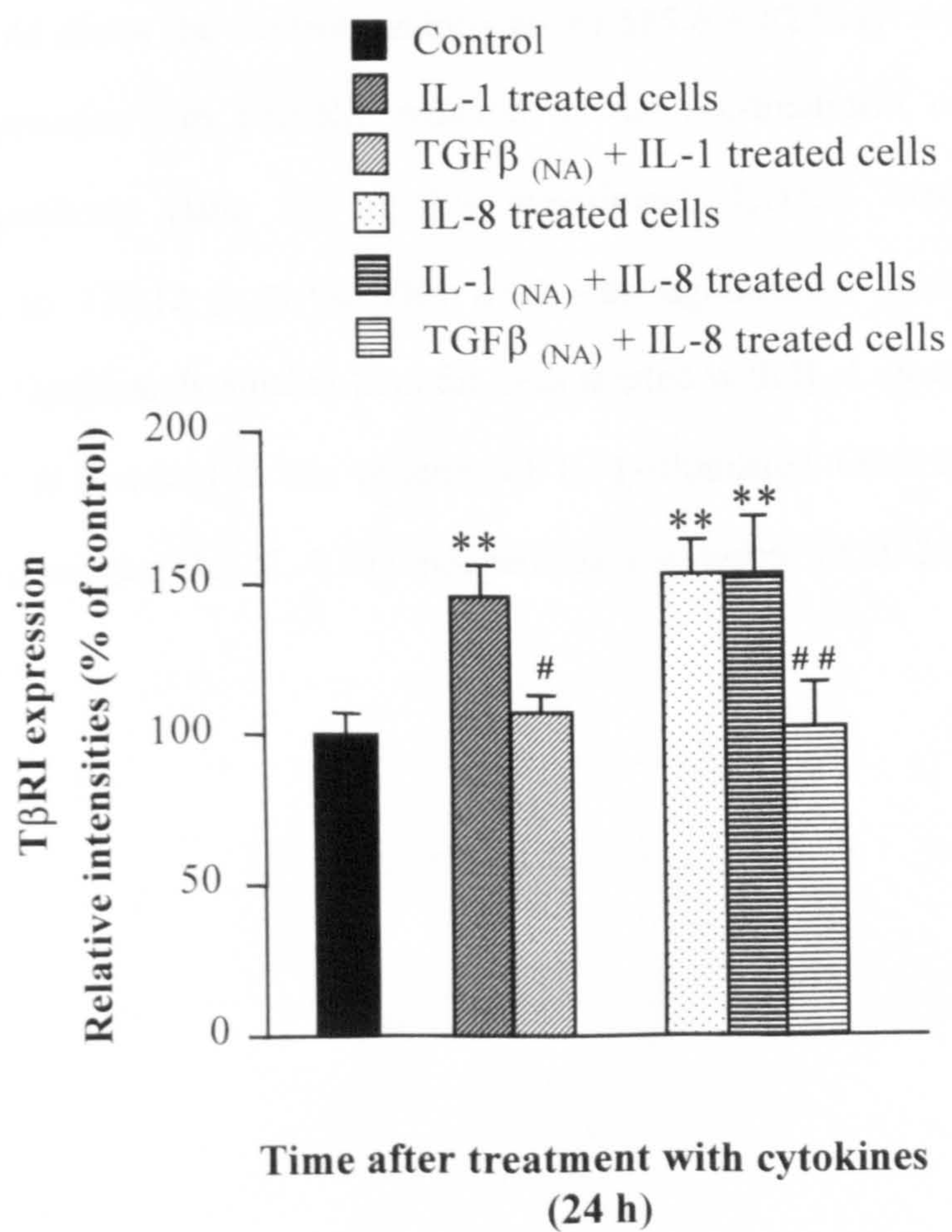
B shows combined analysis of cells from four experiments from four patients. Relative T β RI levels in each group was determined by densitometry. The levels determined for control in each blot (lane 1) were arbitrarily set at 100% and the levels of expression in the others expressed as a change relative to the control level. The values shown are mean \pm SEM.

* indicates a significant difference from the control level and # represents a significant difference from cells treated with either IL-1 or IL-8 alone. #, $P < 0.05$; ** or ##, $P < 0.01$.

A.



B.



5.3 IL-1 stimulation of COX-2 expression

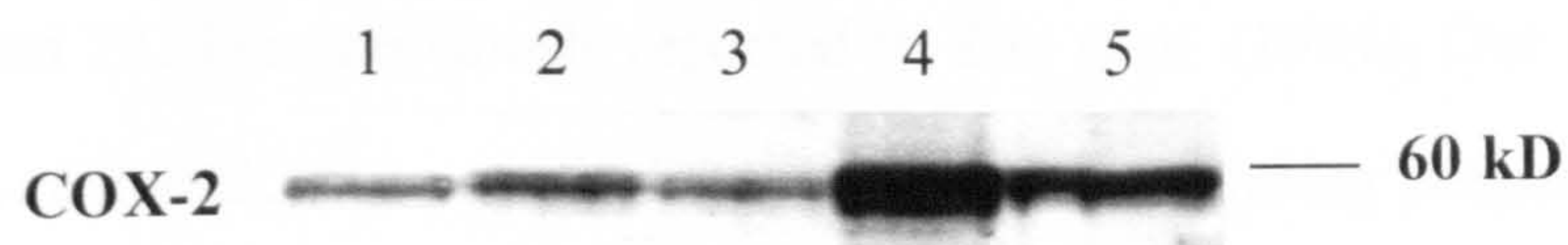
As shown in Figure 5.3A, stimulation with TGF β 1 (lane 2) had little effect on the level of COX-2 expression. In contrast, IL-1 stimulated cells expressed higher levels of COX-2 than those of the control (lane 1) and TGF β 1 treated cells (lane 4). IL-1 induced COX-2 expression was reduced by TGF β 1 neutralising antibody pre-treatment (lane 5). The mean levels of COX-2 expression extracted from four batches of cells isolated from four different samples are shown in Figure 5.2B. Although there was a trend of increasing COX-2 expression in cells stimulated with TGF β 1, the difference was not significant from the control level. In contrast, IL-1 significantly increased COX-2 expression as shown in lane 4 by approximately six-fold above the control: an increase to $585.6 \pm 82 \%$ ($P < 0.001$). IL-1 stimulated COX-2 expression was partially reduced by the pre-treatment of cells with TGF β 1 neutralising antibody (lane 5). In this experiment, COX-2 expression was significantly increased to $375.12 \pm 41 \%$. This level was significantly greater than the control ($P < 0.01$) and significantly smaller than for cells treated with IL-1 alone ($P < 0.05$), indicating that TGF β 1 is involved in the process of IL-1-stimulated COX-2 expression. These observations also suggest that IL-1 can independently stimulate COX-2 expression in human myometrial cells.

Figure 5.3 The effects of IL-1 and TGF β 1 on COX-2 expression in the presence and absence of TGF β neutralising antibody

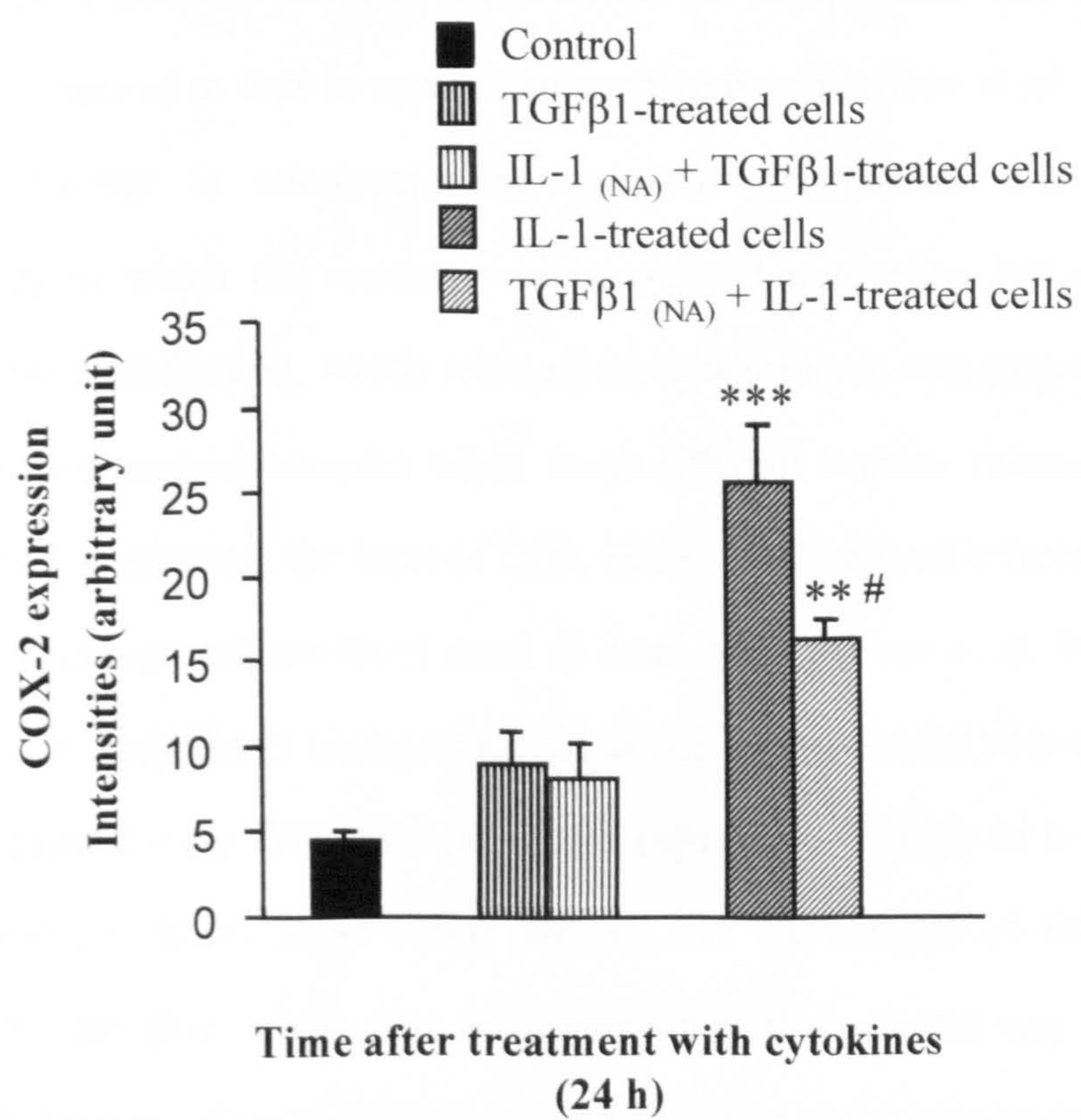
A. Western blots illustrating the expression of COX-2. The individual lanes show: 1. the control level of expression in untreated cells; 2. cells treated with TGF β 1; 3. cells treated with TGF β 1 in the presence of IL-1 neutralising antibody; 4. cells treated with IL-1; 5. cells treated with IL-1 and TGF β 1 neutralising antibody. Neutralising antibodies were added 3 h prior to cytokine addition and were presented throughout cytokine stimulation for 24 h.

B. Combined analysis of cells from 4 separate experiments from four different patients. The levels of COX-2 expression were determined by densitometry. The values shown are mean \pm SEM. * indicates a significant difference from the control level and $^{\#}$ represents a significant difference from cells treated with IL-1 alone. $^{\#}$, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

A.



B.



5.4 Discussion

At term the myometrium is primed for parturition by increasing the expression of proteins essential for cell signalling, SAPs, and contraction, CAPs. COX-2, a regulatory enzyme in the mechanism of PG synthesis, is another example of SAPs whose levels increase with gestational age and decrease with labour (Zuo *et al.* 1994). In the present study it was found that COX-2 in the myometrium was expressed in two bands at approximately 56-60 kD and 72 kD as previously reported by Zuo *et al.* (1994). Our finding that the expression of myometrial COX-2 protein did not change either during pregnancy or during labour was similar to the pattern of COX-2 mRNA and protein expression demonstrated recently (Moore *et al.* 1999; Sparey *et al.* 1999). In contrast, the COX-2 level in this study was different from the previous report that COX-2 expression was decreased at the onset of labour compared to that in non-labouring myometrium (Zuo *et al.* 1994). The reason for this difference is unknown. The previous observation was based on immunohistochemistry in which the scoring was considered qualitative but on Western blotting analysis in this investigation, which relies upon densitometric scanning and state. It has been shown that myometrial samples taken from different regions revealed different trends of COX levels. For instance, the level of COX increased significantly from the uterine fundus toward the lower segment and from outer to inner layer (Keirse *et al.* 1985, Sparey *et al.*, 1999). Thus, it is likely that a topographic difference of expression may occur in the myometrium and account for the difference in COX-2 expression. It must be borne in mind that PG is not stored and is produced when needed, and the half-life of COX is short (Meade *et al.* 1993). Therefore, an increase in expression of this enzyme may be transient and can not be easily detected if the myometrium is not sampled at the appropriate time.

Little is known about the mechanisms by which SAP and CAP genes are activated in myometrial smooth muscle during late pregnancy. However, the findings from chapter 4 suggest that IL-1 and IL-8 can regulate T β RI expression, which is a SAP. There is also indirect evidence to suggest that other SAPs may be regulated by cytokines. For example,

IL-1 has been found to increase PG synthesis in myometrial cells probably by increasing the expression of COX-2 (Todd *et al.* 1996). The data observed in this study show that IL-1 increased the expression of COX-2 in myometrial smooth muscle. This observation raises the possibility that IL-1 could activate genes for T β RI and COX-2 by the same intracellular pathway. However, the results suggest that this is not the case and more complex cytokine interactions are involved in regulating SAPs expression.

Several types of cytokine interaction have been identified in myometrial smooth muscle. Firstly, there may be direct effects, which do not require other cytokines or proteins in the mechanism of action. Examples of this type of interaction may include the stimulation of T β RI by TGF β 1 and the reduction in IL-8 receptor expression by IL-8 as shown in the previous chapter. These examples illustrate positive and negative feedback role of cytokines.

Secondly, the IL-1 stimulation of T β RI illustrates a 'long range' interaction of cytokines. It has been shown in chapter 4 that IL-1 and IL-8 can increase the production of TGF β 1 in human myometrial smooth muscle cells *in vitro*. In addition, TGF β 1 can increase the expression of T β RI. The data from the present study suggest an indirect effect of IL-1 and IL-8 on T β RI expression. The data show that IL-1 and IL-8 stimulation of T β RI is completely inhibited by TGF β 1 neutralising antibody, suggesting that the mechanism of action is mediated exclusively by the sequential production and autocrine action of TGF β 1. It has been hypothesised that a cytokine cascade is involved in the mechanism of normal labour whereby cytokines are produced in a specific sequence. The observations in this study support this hypothesis and demonstrate that in response to IL-1 and IL-8, TGF β 1 is the next cytokine to appear in the sequence of a cytokine cascade in the myometrial cells. The experiments also show that the action of IL-8 is not mediated via the stimulation of IL-1. Therefore it would appear that two cytokines, IL-1 and IL-8, have the same action. The possible synergistic and complex interplay between IL-1 and IL-8 and their effects on T β RI

expression has yet to be elucidated. Other examples of this type of cytokine interaction may include TNF stimulation of PGE₂ production via IL-1 in human osteoblast-like cells (Xu *et al.* 1997).

Thirdly, cytokine interaction may involve a synergistic interplay between cytokines. In the experiment presented here, IL-1 can induce COX-2 expression in myometrial cells, which is consistent with the observation by Hertelendy *et al.* (1999). TGFβ1 does not appear to stimulate this enzyme although a minor increase in the stimulation is observed. However, TGFβ1 acts in conjunction with IL-1 to promote the activation of COX-2 expression. There are many examples of this synergistic action in the literature (Xu *et al.* 1997; Diaz *et al.* 1998). TGFβ, TNF and IL-1, for example, combine to stimulate PGE₂ production in osteoblast-like cells and in human lung fibroblasts (Xu *et al.* 1997; Diaz *et al.* 1998). TGFβ1 alone has no effect on COX-2 expression but the addition of TGFβ1 increases the IL-1 or TNFα stimulation of COX-2 mRNA and protein in human lung fibroblast cells (Diaz *et al.* 1998). The action of TGFβ in lung fibroblasts appears to be via the stabilisation of COX-2 mRNA after the increase in transcription activated by IL-1 (Diaz *et al.* 1998). Overall, a sequential process of cytokine synthesis with autocrine action and a synergistic action between cytokines occurs in myometrial smooth muscle cells *in vitro*.

In addition to interactions between cytokines, the possibility exists that a cytokine may activate a different signalling system, which may then have an effect on the cells. For example, the data presented here show that IL-1 increases TβRI expression. IL-1 also stimulates the production of COX-2 and the release of PG. Thus, the IL-1 induced changes in TβRI expression could be mediated via PG. This idea could be examined directly using an inhibitor of COX-2 to remove any effect of increase PG. This was not done in the present study. Indirect evidence suggests that such an interaction may not occur in the myometrium. Prostaglandin can stimulate cAMP formation (LopezBernal *et al.* 1991; Europe Finner *et al.* 1994) and cAMP dramatically reduced TβRI expression (see chapter 6). Thus, the action of

prostaglandin, mediated through cAMP, would be predicted to give an opposite result to that observed.

In conclusion, these in vitro results may illustrate the complexities of an autocrine-signalling cascade within the myometrial smooth muscle in the physiological process of the maturation of myometrium. It would appear that the autocrine cascade regulates the expression of specific SAPs. Specific cytokines may also be the primary activators of SAP and CAP expression. IL-1, for example, is the primary activator of COX-2 while TGF β 1 is the primary activator of RyR2 (Awad *et al.* 1997) and T β RI. In addition to operating at the level of gene expression, cytokines may also influence the post-translational regulation of proteins. If this idea is correct and individual cytokines are dominant at different stages in the cascade then different SAPs and CAPs will appear at different times in late pregnancy. The synergistic actions of cytokines may further fine-tune the expression of SAPs and ultimately the expression of CAPs. Consequently, the development of contractility occurs within the myometrium at term in preparation for parturition.

Chapter 6

Effect of raised cAMP on TGF β 1 and T β RI Expression in human myometrial cells *in vitro*

The mechanisms regulating SAP and CAP expression are poorly understood. From our previous study, a complex cytokine-signalling cascade has been suggested to be involved in this mechanism in the latter stages of pregnancy. TGF β 1 and TGF β receptors are part of this cytokine-signalling cascade in myometrial smooth muscle cells. Factors regulating the expression of TGF β 1 and T β RI are not clearly understood but, at least in part, involve other cytokines such as IL-1 and IL-8 and sex steroid hormones as described in the previous chapters.

It has been recognised that progesterone is important for maintaining myometrial quiescence throughout pregnancy (Liu & Rebar, 1999). Progesterone acts through a number of mechanisms including inhibition of prostaglandin production and CAP synthesis (Able *et al.* 1980; Lye, 1996). Genes encoding for proteins that are involved in cAMP production have been known to regulate myometrial quiescence in mid-pregnancy and myometrial contraction at term (Europe Finner *et al.* 1993, 1994). cAMP activates protein kinase A to promote smooth muscle relaxation (Sanborn *et al.* 1998). cAMP has been shown to be involved in the regulation of gene expression for example TGF β 1 in pituitary and choriocarcinoma cells (Ritvos & Eramaa, 1991; Pastorcic & Sarkar, 1997) and prolactin (Peers *et al.* 1991). A role for cAMP in gene regulation in human myometrial smooth muscle cells has yet to be established

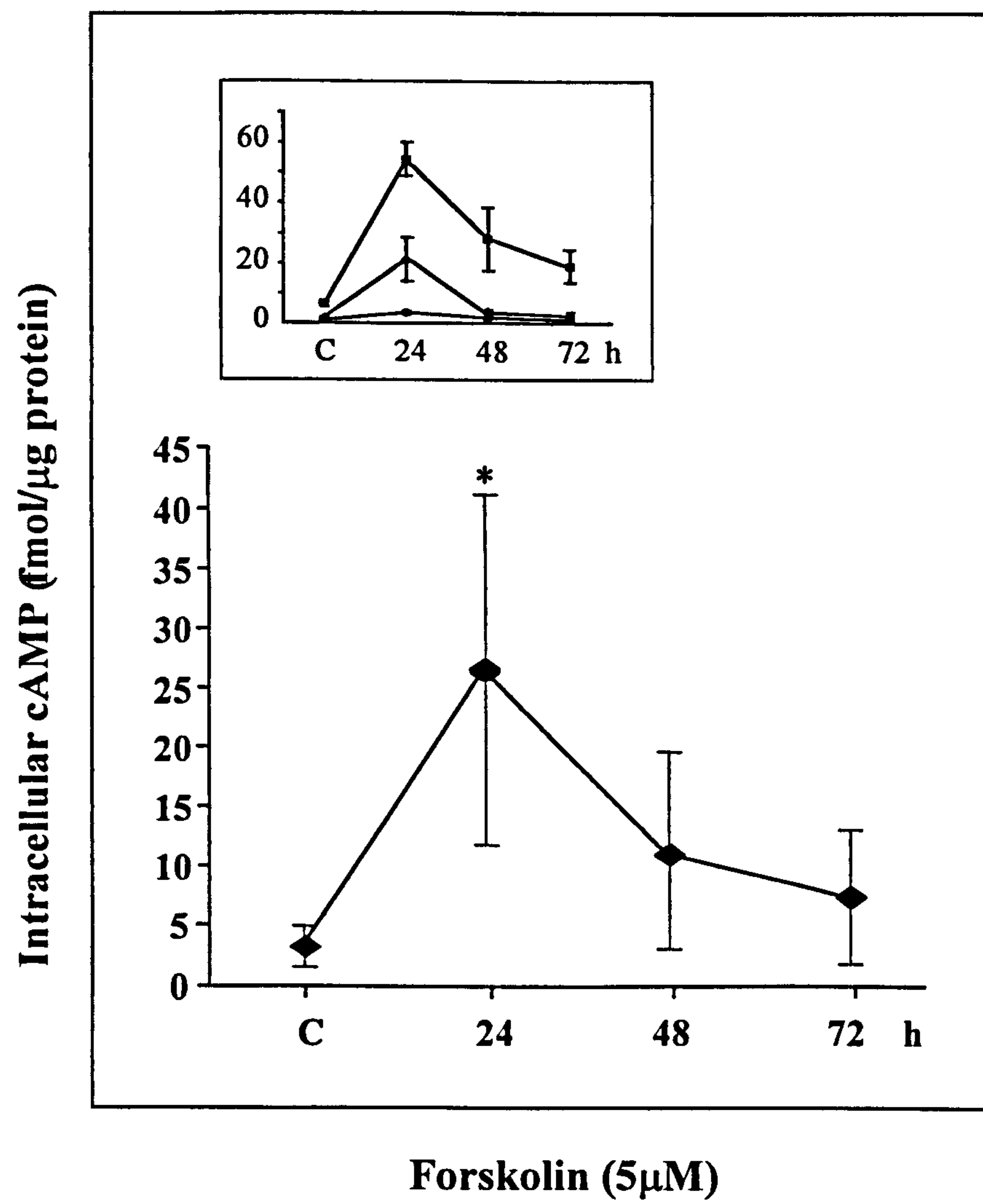
It can be speculated that changes in the level of cAMP may be involved in triggering or modulating the autocrine-signalling cascade in the myometrium during pregnancy. In this study the effects of increasing cAMP by forskolin, an activator of adenylyl cyclase, on the expression of TGF β 1 and T β RI were determined in order to examine this hypothesis

6.1 Forskolin effect on cAMP levels in myometrial cells *in vitro*

The first series of experiments were done to confirm that exposing human myometrial cells to forskolin resulted in an elevation in the level of intracellular cAMP. Figure 6.1 (inset within the main panel) shows the response of three individual cells isolated from three different subjects (performed in triplicate). Although it was noted that there were differences in responses to forskolin in individual batches, their responses were similar in trend when compared to those of control cells. As shown in the main panel, treatment of cells with forskolin (5 μ M) for 24 h slightly increased cAMP from a basal level of 1.68 ± 0.89 to 26.19 ± 7.84 fmol/ μ g protein ($P < 0.05$). Treatment with forskolin for 48 and 72 h did not result in a significant rise in cAMP: 11.22 ± 5.25 and 7.34 ± 3.21 fmol/ μ g protein respectively. Thus, the effect of prolonged forskolin treatment on the elevation of intracellular cAMP is transient. The present data show that after 24 h of forskolin treatment, cAMP still remains elevated. The effect of forskolin treatment on TGF β receptor is clear (see the next section). However, the precise time at which the elevated cAMP interacts with the genome to produce this effect can not be discerned from the present experimental protocol. This, however, does not detract from the observation of the profound effect on the TGF β receptor expression. A decrease in the levels of cAMP after 48 and 72 h may reflect the down regulation of adenylyl cyclase during prolonged stimulation or the degradation of cAMP by phosphodiesterase as no enzyme inhibitor was applied in this experiment.

Figure 6.1 Elevation of intracellular cAMP by forskolin

Confluent human myometrial smooth muscle cells were cultured in the presence of forskolin (5 μ M) and without forskolin for the indicated time (C, control at 24 h). Following the treatment, cAMP content in the cells was measured using cAMP enzyme-immunoassay. The data shown in an inset panel is the data from three different experiments performed in triplicate on cells from three myometrial samples. The data shown in the main panel are the mean data from three samples. Values at each time point are mean \pm SEM and expressed as fmol/ μ g protein. *, $P < 0.01$ as compared to the control.



6.2 Forskolin effect on TGF β 1 and T β RI expression

Figure 6.2 shows data obtained using ELISA to measure the total levels of TGF β 1 in the supernatant of control cultures and cells exposed to forskolin for progressively longer periods of time. The steady state levels of TGF β 1 production were increased significantly from control levels of 23.53 ± 0.61 pg/ μ g protein to 29.89 ± 1.08 pg/ μ g protein at 72 h of treatment. At 24 and 48 h there was no significant difference in the total level of TGF β 1 in cells treated with forskolin compared to the control cells. Since cAMP is not elevated at 72 h, it is doubtful that the small increase represents a true response.

The effects of prolonged exposure to forskolin on T β RI appeared more complex than the stimulation of TGF β 1. Figure 6.3A shows an example of a Western blot illustrating T β RI expression in one batch of cells. The level of expression in control cells is shown in lane 1. This level was arbitrarily set to 100 % in order to compare the levels of expression in the treated cells. Exposing cells to forskolin for 24 h reduced T β RI expression (lane 2). After 48 or 72 h the levels of expression were greater than the level measured at 24 h and did not appear to be significantly different from control levels (lanes 3 and 4). Figure 6.3B shows mean data from five separate experiments on five batches of myometrial cells. Raising cAMP for 24 h decreased T β RI expression significantly to 9.7 ± 2.29 % compared to control levels ($P < 0.001$). With 48 and 72 h treatments T β RI expression was 74 ± 14.51 % and 102.6 ± 10.14 % compared to control levels. The levels of expression at 48 and 72 h were significantly greater than at 24 h but did not appear to be significantly different from the control level.

Figure 6.2 The effect of forskolin on TGF β 1 levels

Confluent human myometrial smooth muscle cells were cultured in the presence of forskolin (5 μ M) and without forskolin for the indicated time (C, mean data of control at 24, 48 and 72 h). Following the treatment the total concentration of TGF β 1 in the culture medium was measured using ELISA. Values are mean \pm SEM of four experiments from four different tissues assayed in duplicate (n = 4) and expressed as pg/ μ g protein. **, $P < 0.01$ as compared to the control.

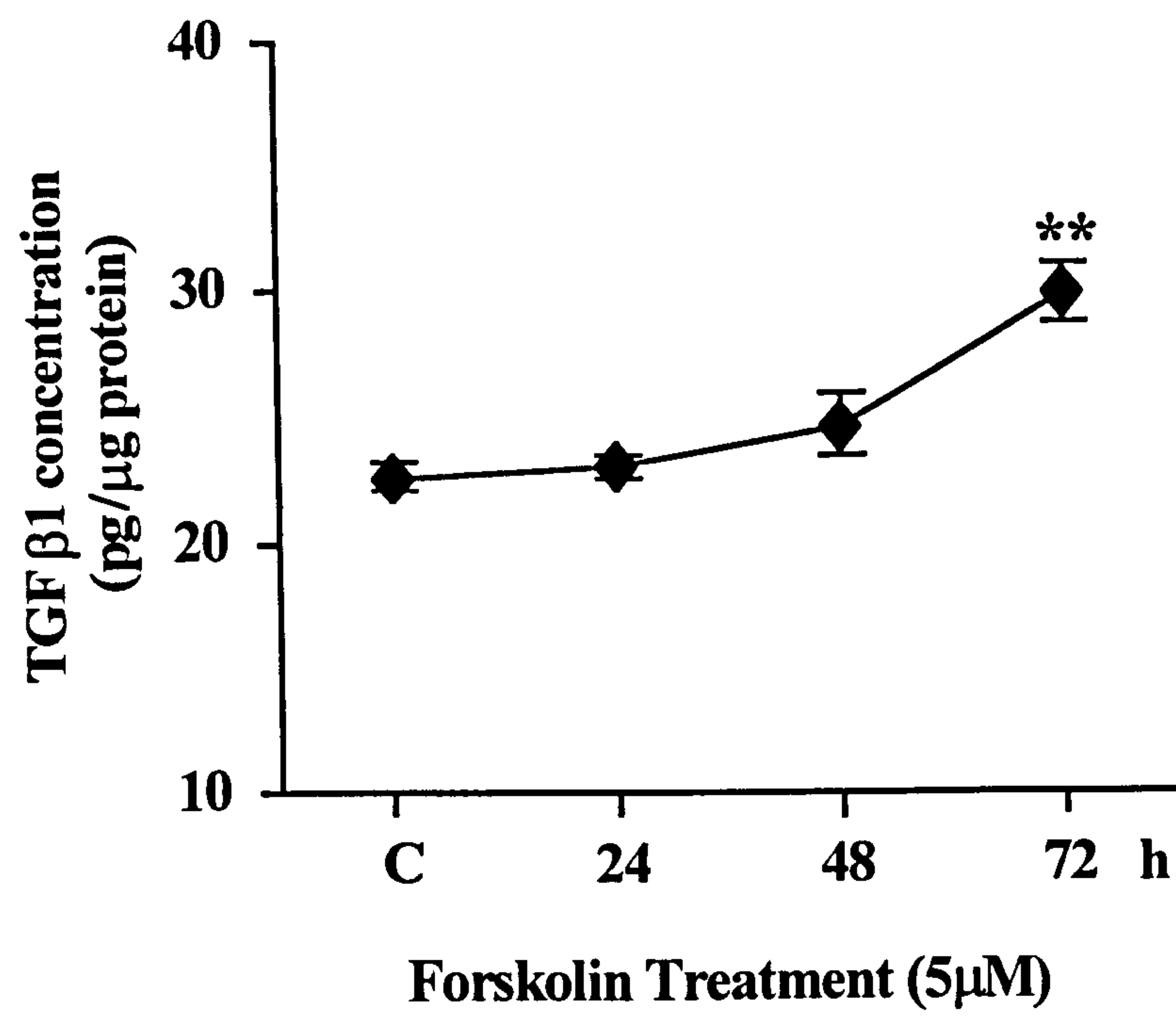
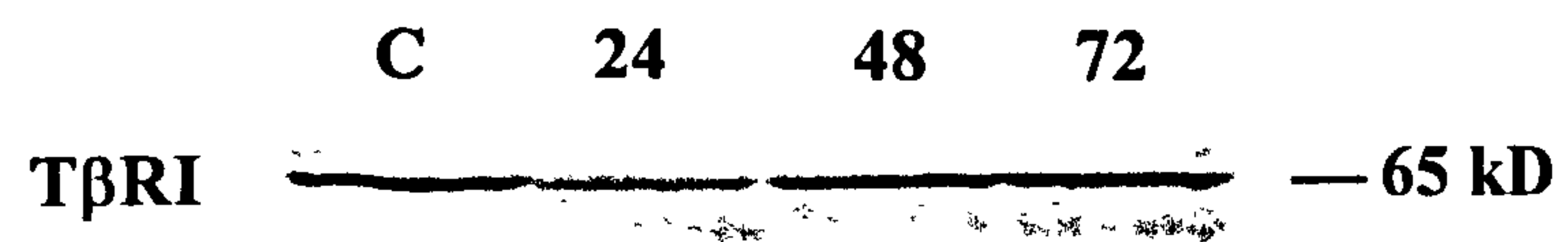


Figure 6.3 The effect of forskolin on the expression of T β RI

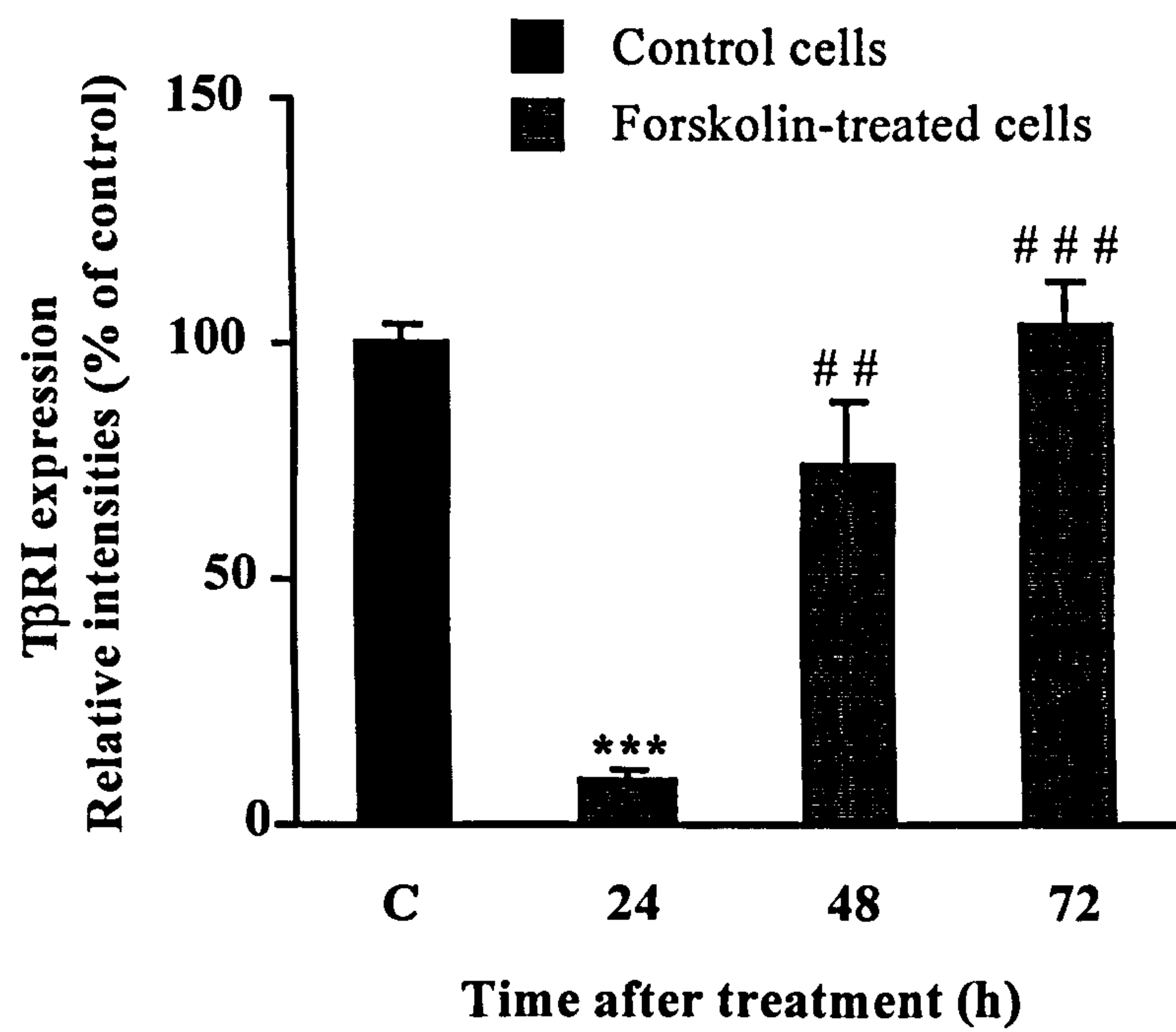
Myometrial smooth muscle cells (40 μ g) from control (C) and cells treated with forskolin (5 μ M) were separated in 7.5 % SDS-PAGE and subjected to Western blot analysis with antibodies to T β RI. A shows an example of T β RI expression in cells: control at 24 h (lane 1), forskolin treated cells for 24, 48 and 72 h (lane 2, 3 and 4, respectively).

B illustrates analysis of five experiments isolated from five different samples (n = 5). Relative T β RI levels in each blot were determined by densitometry. The levels determined in each blot for the control cells were arbitrarily set at 100% and the levels of expression in the forskolin treated cells expressed as a change relative to the control level. The values shown are mean \pm SEM. ***, $P < 0.001$ as compared to the control; **, $P < 0.01$; ***, $P < 0.001$ as compared to treated cells at 24 h.

A.



B.



6.3 The effects of TGF β 1 on G α_s and G $\alpha_{i1,2}$ expression

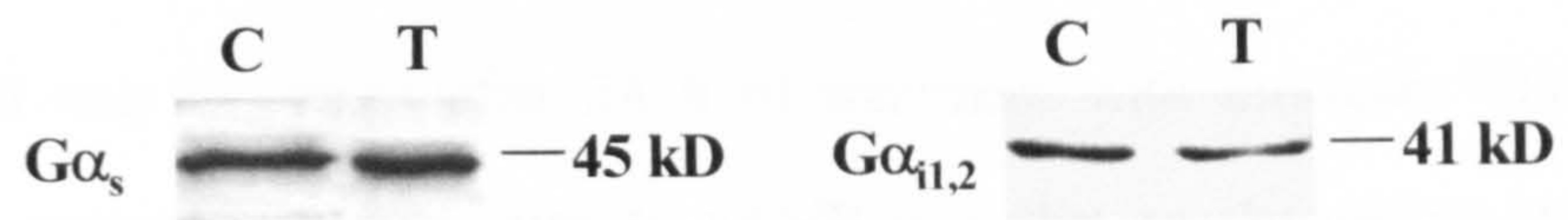
The data are consistent with the operation of a signalling cascade with cAMP feeding forward to regulate the expression of T β RI. In late pregnancy during labour the expression of the G-protein (G α_s), which regulates adenylyl cyclase and cAMP levels, is decreased (Europe Finner *et al.* 1994). With such a sequence of signalling events, it is possible that TGF β 1 in the myometrium might feed back on the cAMP system by affecting the ability of the smooth muscle cells to produce cAMP. Therefore, a series of experiments was carried out to determine the effect of exposing myometrial cells to TGF β 1 (1 ng/ml) for 24 h on the expression of G protein sub-units that stimulate and inhibit adenylyl cyclase activity.

Figure 6.4A illustrates examples of Western blots showing the levels of G α_s detected at 45 kD and G $\alpha_{i1,2}$ at 41 kD respectively in control cells and cells treated with TGF β 1. Figure 6.4B shows mean data from densitometric scans of the Western blots from five preparations of cells from different patients for G α_s and four preparations for G $\alpha_{i1,2}$. There was no significant change in the expression of both G α_s and G $\alpha_{i1,2}$ in the TGF β 1 treated cells. Thus, there is no evidence for a feedback mechanism of TGF β 1 on G-proteins under these conditions.

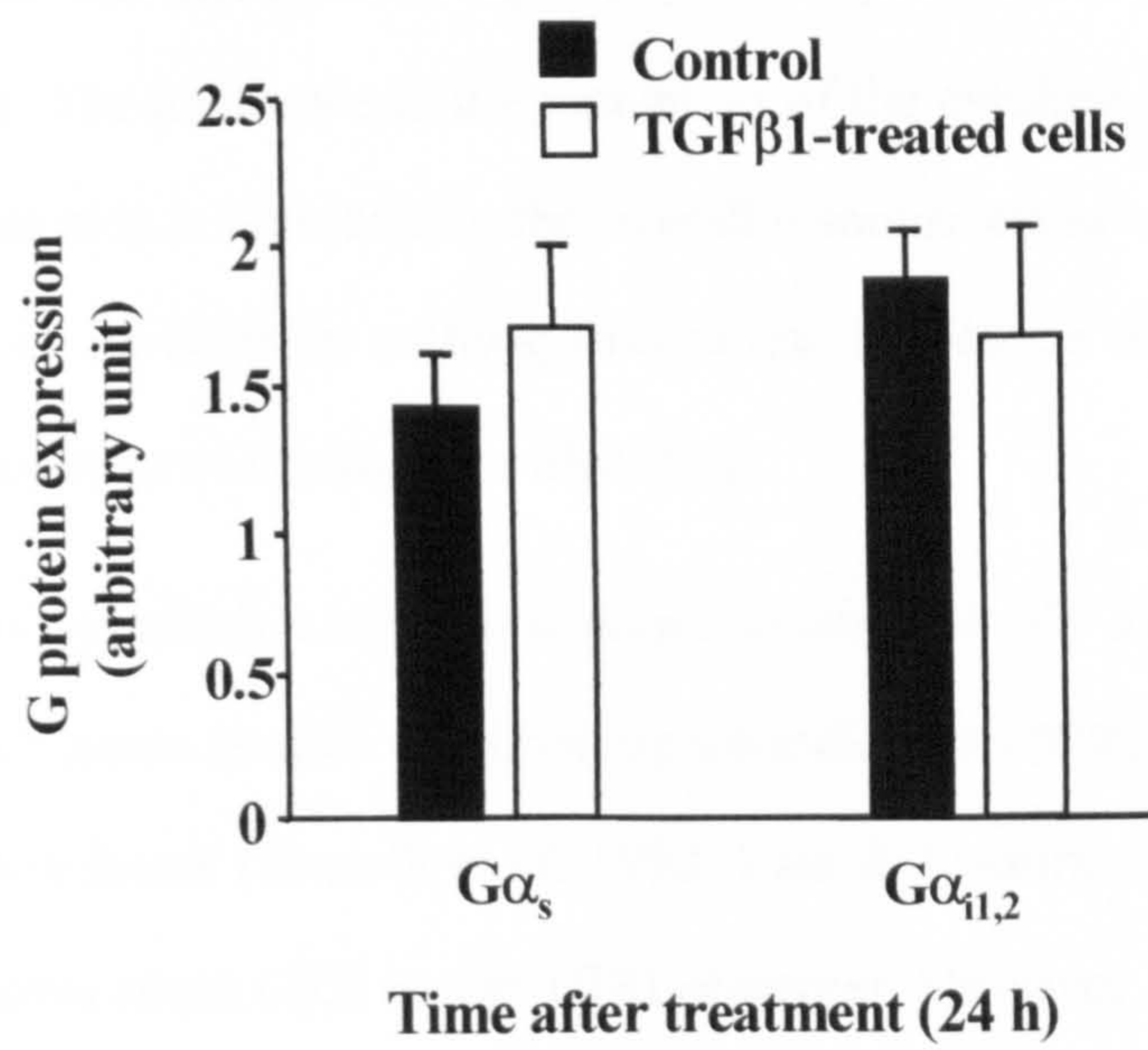
Figure 6.4 The effects of TGF β 1 on the expression of G α_s or G $\alpha_{i1,2}$

A illustrates examples Western blots showing the levels of G α_s and G $\alpha_{i1,2}$ in control cells and cells treated with TGF β 1 (1 ng/ml) for 24 h. B shows mean data \pm SEM from densitometric scan of the Western blots from five preparations of cells from different patients for G α_s (n = 5) and four preparations from four different batches for G $\alpha_{i1,2}$ (n = 4). The values shown are expressed in arbitrary unit.

A.



B.



6.4 Discussion

The present study has been carried out in a timescale from 24 h to 72 h. The fact that the cAMP is not elevated at 48 and 72 h after forskolin treatment means that is difficult to interpret the consequences of the treatment. For this reason the discussion of the data derived from 24 h treatment is considered to be more relevant. The results confirm that treatment with forskolin for periods up to 24 h causes an increase in cAMP levels. Expression of T β RI was decreased after 24 h of treatment with forskolin. Thus, the expression of T β RI appears to be regulated by cAMP such that an elevated cAMP level reduces its expression.

In contrast, an elevation in cAMP at 24 h does not affect the production of TGF β 1. It can be suggested that the mechanisms regulating the expression of the cytokine and its receptor are different. The difference in the regulation of the cytokine and its receptor may represent an important step in determining the overall responsiveness of the myometrium *in vivo*. The nature of the intracellular pathways regulating expression of the elements of the TGF β system in myometrium remains to be identified.

The mechanisms by which cAMP modulates myometrial cell T β RI are not known. Most cAMP-mediated genes contain cAMP-responsive elements (CRE) that are recognised by CREB transcription factor (Borrelli *et al.* 1992; Lalli & Sassone-Corsi, 1994). To date there is no data available about CRE on the T β RI promoter. However, study by Bloom *et al.* (1996) indicates that the human T β RI promoter contains the Sp1 binding sites. cAMP has been shown to modulate transcription factor Sp1 in leukaemia cells (Rohlf *et al.* 1997). Therefore it is possible that cAMP may modulate the activity of transcription factor Sp1 that binds to the Sp1 binding sites on the T β RI promoter. This may result in decreased T β RI expression at 24 h as shown in this study. The transient reduction of T β RI expression may also result from post-transcriptional mechanisms such as the stabilisation of mRNA or enhanced receptor degradation.

It has been reported that an increase in $G\alpha_s$ expression in human myometrium during gestation favours myometrial relaxation by increasing agonist-induced cAMP production (Europe Finner *et al.* 1993). A decrease in this G-protein sub-unit may trigger the mechanism for initiation of labour (Europe Finner *et al.* 1994). Although cAMP has been proposed to dominate the quiescent phase of pregnancy, the intracellular levels of cAMP have not been measured directly. As term approaches the relaxing actions of exogenous isoproterenol stimulated adenylyl cyclase decrease without any change in enzyme activity stimulated by forskolin. This may be the result of a decrease in $G\alpha_s$ coupling to adenylyl cyclase towards term (Litime *et al.* 1989) or a decrease in the expression of β -adrenergic receptors at the end of pregnancy (Breuiller *et al.* 1987). This event may lead to a decrease in cAMP production. During pregnancy, the level of $G\alpha_s$ expression in myometrium changes but not the level of adenylyl cyclase (Europe Finner *et al.* 1994). Taken together these observations suggest that any regulation of cAMP may be at the level of agonist concentrations or the expression of membrane receptors coupled to G protein rather than the changes in adenylyl cyclase levels.

In this study, the reciprocal situation does not appear to occur, in which TGF β 1 does not have any regulatory influence of the expression of G-protein sub-units that regulate cAMP production. Therefore, it can be suggested that there is not a negative feedback from TGF β 1 to decrease G protein expression in cultured myometrial smooth muscle cells and possibly in the myometrium *in vivo*.

In summary, the present study demonstrates the cAMP-induced decrease in T β RI expression in the myometrial smooth muscle cells. The significance of this observation is not clearly understood. cAMP favours myometrial relaxation during pregnancy and TGF β 1 has been reported to be involved in the regulation of CAP. Based on these assumptions, it is possible that increased cAMP during early and mid pregnancy may reduce the responsiveness of cells to TGF β 1 and that results in maintaining myometrial quiescence. It

is clearly that further study of functional consequence of the observed change in T β RI expression may lead to better understanding in the physiological role of TGF β 1 system in the myometrium.

Chapter 7

CONCLUSIONS

In the aim of this thesis the following hypotheses were proposed

- 1). *'that TGFβ1 and TGFβ receptors are present in the myometrium at term'.*
- 2). *'that expression changes with gestation'.*

The results show that TGFβ1 and TGFβ receptors are present in the myometrium at term, confirming the first hypothesis. The expression of TGFβ1 does not change dramatically with gestation. However, TGFβ receptor expression changes with gestation, supporting the second hypothesis.

The results obtained from this study have shown that TGFβ1 and TβRI can be modulated by cytokines, sex steroids and cAMP. This demonstrates that regulation of TGFβ1 and its receptor in myometrial cells and possibly in the myometrium *in vivo* is complex. In addition, the fact that myometrial cells can produce TGFβ1, suggests an autocrine role in the myometrium. The finding that the expression of the cytokine receptors changes with pregnancy has led us to introduce the concept of signalling-associated proteins (SAPs). The SAPs are responsible for the regulation of other SAPs that trigger the expression of CAPs. This introduces the further concept of autocrine cytokine interaction in the myometrium leading to the preparation of myometrium for term.

The onset of labour begins when SAPs and CAPs are in place at the right time. If SAPs and CAPs are expressed before term, this may lead to myometrial activation resulting in premature delivery. If SAPs and CAPS are not in place, successful labour will be unable to proceed. TGFβ1 and TβRI are SAPs and TGFβ1 is known to up-regulate RyR2 in myometrial cells. It is known that TGFβ1 can induce gene expression through TβRI activation. Thus, the functional role of TGFβ1 as a direct stimulator for other CAPs has yet to be elucidated. The importance of TGFβ1 in human parturition, apart from being a CAP

stimulator, has also been suggested in the literature. For example, Casey & MacDonald (1997) suggested that TGF β 1 acts in myometrial cells to negate some actions of progesterone on the expression of myometrial Cx-43 and endothelin-1. This idea shows an alternative mechanism of progesterone withdrawal, which is not present in human pregnancy.

In this thesis, cultured myometrial cells maintained in fetal calf serum have been used. Cultured cells have both advantages and disadvantages. The disadvantages are that culture model is an isolated system and consequently the environment of the cells must be provided by the experimenter. In consequent the choice of the media is inevitably arbitrary. In addition, as a consequence of cells being *in vitro* they are prone to differentiation and phenotypic change that might not represent cells *in vivo*. However, cultured cells allowed us to manipulate the environment, which can not be done in humans, to obtain information regarding how the cells are regulated.

From the overall observations in this thesis, the following model (Figure 7.1) for the regulation of T β RI, leading to preparation of human myometrium can be proposed.

Directions for future work

1. To determine the time course of other cytokines and cytokine receptors during the last trimester of pregnancy
2. To determine the relationship between the expression of CAPs and the expression of CAPs and CAPs
3. To identify the signalling pathways of TGF β 1 which affects the expression of TBRI

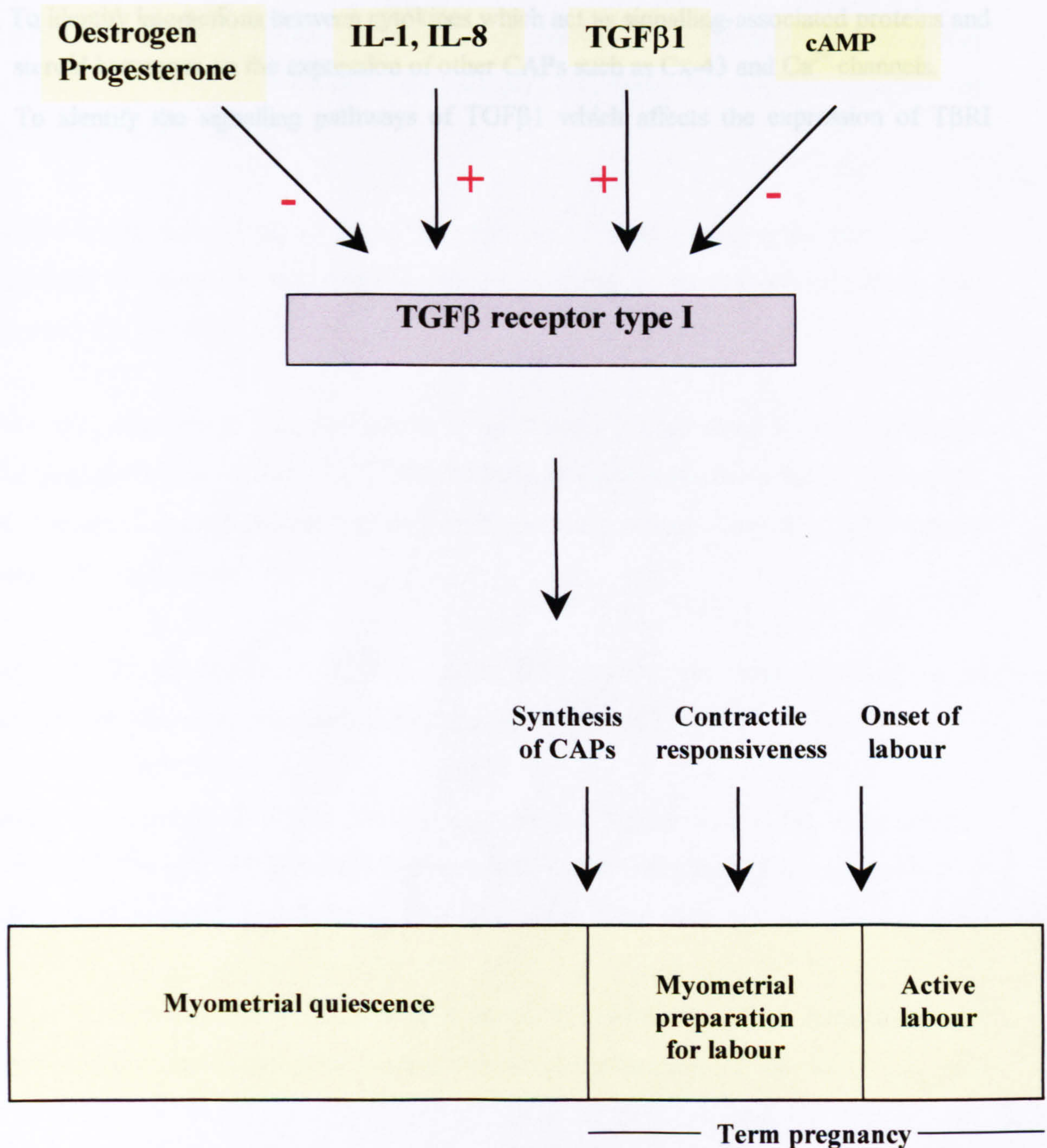


Figure 7.1 Proposed model for the preparation of the human myometrium

Directions for future work

1. To determine the time course of other cytokines and cytokine receptors during the last trimester of pregnancy.
2. To identify interactions between cytokines which act as signalling-associated proteins and steroid hormones on the expression of other CAPs such as Cx-43 and Ca^{2+} channels.
3. To identify the signalling pathways of $\text{TGF}\beta 1$ which affects the expression of $\text{T}\beta\text{RI}$

Chapter 8

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Appendix 1

Abbreviation

AP-1	Activator protein-1
BSA	Bovine serum albumin
[Ca²⁺]_i	Intracellular free calcium concentration
cAMP	Adenosine 3'5'-cyclic monophosphate
CAP	Contraction Associated Protein
COX-2	Cyclooxygenase-2
Cx-43	Connexin-43
DAB	Diaminobenzidine
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
FCS	Foetal calf serum
G-protein	Guanine nucleotide binding protein
HBSS	Hanks balanced salt solution
HEPES	N-2-hydroxyethylpiperazine-N-2-ethan- sulphonic acid
HRP	Horseradish peroxidase
IL-1	Interleukin-1
IL-8	Interleukin-8
IL-1RI	Interleukin-1 receptor type I
IL-8RB	Interleukin-8 receptor type B
kD	Kilodalton
mRNA	Messenger ribonucleic acid
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PG	Prostaglandin
RyR	Ryanodine receptor
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SAP	Signalling Associated Protein
TBS	Tris buffered saline

TGFβ	Transforming growth factor beta
TβRI	TGFβ receptor type I
TβRII	TGFβ receptor type II
TβRIII	TGFβ receptor type III
Tris	2-amino-2-(hydroxymethyl)-1,3-propandiol

Appendix 2

List of suppliers

Amersham Life Science Ltd.

Amersham Place,
Little Chalfont,
Buckinghamshire,
HP7 9NA
Tel 0800 515313

Bio-Rad Laboratories

Bio-Rad House,
Mayland Avenue,
Hemel Hempstead,
Hertfordshire,
HP2 7TD
Tel 0800 181134

Dako Ltd.

Denmark House,
Angel Drove, Ely,
Cambridgeshire,
CB7 4ET
Tel 0135 366 9911

Insight Biotechnology Ltd.

PO Box 520,
Wembly,
Middlesex,
HA9 7YN
Tel 0181 385 0303

NENTM Life Science Products, Inc.

UK
Tel 0800 896046

R&D Systems Europe Ltd.

4-10 The quadrant,
Bartaon Lane
Abingdon
OX14 3YS
Tel 0123 555 1100

Schleicher & Schuell UK Ltd.
Unit 11, Brunswick Park Industrial Estate,
London,
N11 1JL
Tel 0181 361 3111

Sigma-Aldrich Company Ltd.
Fancy Rd,
Poole,
Dorset
BH12 4QH
Tel 0800 717181

Whatman International Ltd.
Whatman House,
8t Leonard's Rd,
20/20 Maidstone,
Kent,
ME18 0LB
Tel 0162 267 4821

Appendix 3

Solution compositions for SDS-PAGE

Acrylamide gel, 30 %	30 g acrylamide, 0.36 g bis-acrylamide in 100 ml distilled water
Separating buffer	1.5 M Tris base, pH 8.8 and 0.4 % SDS (adjust pH with HCl)
Stacking buffer	0.5 M Tris base, pH 6.8 and 0.4 % SDS (adjust pH with HCl)
Gel running and electrophoresis	25 mM Tris base, pH 8.6, 192 mM Glycine and 0.1 % SDS

Formulation for separating gel (7.5 %)

Acrylamide gel	2.2 ml
Separating buffer	2.25 ml
Distilled water	4.03 ml
TEMED	7.5 µl
Ammonium persulfate (15 mg/ml)	225 µl

Formulation for stacking gel (5 %)

Acrylamide gel	0.84 ml
Stacking buffer	1.25 ml
Distilled water	2.75 ml
TEMED	5 µl
Ammonium persulfate (15 mg/ml)	125 µl

Solution composition for transfer buffer

Transfer buffer (10 x)	0.25 M Tris base, pH 8.6 and 1.92 M Glycine
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Formulation for working transfer buffer (100 ml)

Transfer buffer (10 x)	10 ml
Methanol	20 ml
Distilled water	70 ml

Appendix 4

Components of TGFβ1 assay system

Micro-titre plate	96 wells coated with mouse anti-TGFβ1
Detection antibody	rabbit antibody against TGFβ1, 75 μl
Biotinylated second antibody	anti-rabbit Ig conjugated to biotin, 50 μl
Streptavidin-HRP conjugate	streptavidin conjugated to horseradish peroxidase
Standard	recombinant TGFβ1 in an acid/gelatin buffer, which give 21 ng/ml after reconstitution
Standard reconstitution reagent	2 ml of 4 mM HCl and 0.1 % BSA
Assay buffer	15 ml of concentrate buffer, when diluted with PBS (1:10), gives 0.01 M PBS, pH 7.4 containing 0.1% BSA (w/v)
Wash buffer	15 ml of concentrate buffer, when diluted with PBS (1:100), gives 0.01 M PBS, pH 7.4 containing 0.2 % (v/v) Tween-20
PBS tablet	0.01 M PBS, pH 7.4 (one tablet dissolved in 200 ml of distilled water)
TMB substrate	3,3', 5,5'-tetramethylbenzidine/hydrogen peroxide solution in 20 % (v/v) dimethylformamide, 22 ml
Stop solution	0.19 M sulfuric acid, 12 ml

Reagents for sample activation

1 M HCl (100 ml)	add 10 ml of concentrated (10 M) HCl to 90 ml of distilled water
1.2 M NaOH/0.5 M HEPES (100 ml)	4.8 g NaOH dissolved in 75 ml of distilled water. Add 11.9 g of HEPES and adjust volume to 100 ml with distilled water

Appendix 5

Components of cAMP assay system

Micro-titre plate	96 wells coated with donkey anti-rabbit IgG
Assay buffer	0.05 M sodium acetate buffer, pH 5.8 containing 0.02 % BSA and 0.01 % preservative
Standard (for non-acetylate assay)	cAMP standard, on reconstitution gives 32 pmol cAMP/ml
Antibody	Rabbit anti-cAMP
Peroxidase conjugate	cAMP-horseradish peroxidase
Wash buffer concentrate	on dilution give 0.01 M PBS, pH 7.5 containing 0.05 % Tween-20
TMB substrate	3,3', 5,5'-tetramethylbenzidine/hydrogen peroxide solution in 20 % dimethylformamide
Lysis reagent 1	2 g dodecyltrimethylammonium bromide
Lysis reagent 2	5 g of solid contain no hazardous chemical

Reagent preparation (all reagents were equilibrated to room temperature before used)

Assay buffer	dilute with 500 ml of distilled water
Lysis reagent 1A	dissolve 2 g of lysis reagent 1 in 80 ml of assay buffer
Lysis reagent 1B	take 10 ml of lysis reagent 1A and make up to 100 ml with assay buffer
Lysis reagent 2A	dissolve 5 g of lysis reagent 2 in 100 ml of assay buffer
Lysis reagent 2B	take 10 ml of lysis reagent 2A and make up to 40 ml with assay buffer
Standard	reconstitute in 2 ml of lysis reagent 1B, the final solution contains 32 pmol cAMP/ml
Antiserum	dilute with 11 ml of lysis reagent 2B
cAMP peroxidase conjugate	dilute with 11 ml of assay buffer
Wash buffer	dilute with 500 ml of distilled water